FREE RADICAL FORMATION AND DNA DAMAGE IN THE CYTOTOXICITY OF ALKYLAMINOANTHRAQUINONE ANTITUMOUR AGENTS.

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Declaration

The work reported in this thesis is original except where due reference is made and has not been submitted in whole or in part for any other degree of the C.N.A.A. or for any degree of any other degree awarding body or institution.

G. R. Fisher.

Geoffrey Roy **FISHER**

Abstract

Free radical formation and DNA damage in the cytotoxicity of alkylaminoanthraquinone antitumour agents.

Geoffrey Roy FISHER

The role of NAD(P)H dependent reductase mediated redox cycling in DNA strand breakage and cytotoxicity by doxorubicin (DOX), mitozantrone (MIT), CI941 and a series of alkylaminoanthraquinones (AQ) based on MIT has been investigated in MCF-7 human breast cancer cells. 1AQ, 1,8AQ and DOX were found to redox cycle in MCF-7 cells as evidenced by stimulation of NADPH oxidation, superoxide anion formation and hydroxyl radical formation associated with generation of a drug free radical in MCF-7 S9 cell fraction. Furthermore, DOX formed a free radical, stimulated oxygen consumption and produced hydroxyl radicals in intact viable MCF-7 cells. 1,5AQ formed a free radical species and produced hydroxyl radicals but did not stimulate NADPH oxidation or superoxide anion formation in MCF-7 S9 fraction. Reactive oxygen formation correlated with the DNA strand breakage and cytotoxicity produced by 1AQ, 1,5AQ, 1,8AQ and DOX in MCF-7 cells. DOX also produced plasmid DNA strand breakage in the presence of purified cytochrome P450 reductase or xanthine oxidase. 1AQ, 1,5AQ and 1,8AQ mediated plasmid DNA strand breakage only in the presence of purified cytochrome P450 reductase. MIT, CI941 and 1,4AQ did not redox cycle in MCF-7 cell S9 fraction yet produced equivalent cellular DNA strand breakage to DOX and AQ's at LD50 concentrations. The order of cytotoxicity of these compounds after a 1 hour exposure and at least 6 days further cell growth was;- (LD50);- CI941(1.5×10^{-10}M) >MIT(5.2×10^{-9}M) >1AQ(6.0×10^{-8}M) >1,8AQ(0.5×10^{-6}M) >1,4AQ(1.2×10^{-6}M) >DOX(3.0×10^{-6}M) >1,5AQ(12.3×10^{-6}M). Variation in cellular uptake between the agents did not account for differences in cytotoxicity and DNA strand breakage observed. The 1,4 substitution pattern of MIT, CI941 and 1,4AQ appears to prevent metabolic activation. In further studies;- i) MIT and CI941 were more potent inhibitors of MCF-7 topoisomerase I activity than other quinones, ii) MIT and DOX-Fe^{3+} complexes mediated plasmid DNA strand breakage in the presence of reduced glutathione iii) Only MIT was oxidatively activated by horseradish peroxidase/H_2O_2, producing plasmid DNA damage. The results indicate that future development of quinone antitumour agents should concentrate on compounds which do not redox cycle but have functional groups that assist iron binding, activation by peroxidases and/or topoisomerase inhibition. In this respect, these agents should possess chromophore hydroxyl groups as these appear to be responsible for the increased cytotoxicity of MIT and CI941 compared to the other AQ's.

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CHAPTER 1

Introduction

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Introduction

1.1 Cancer

Cancer is a major disease worldwide, with an estimated 5.9 million cases occurring throughout the globe each year. One half of these cases involve cancer of the stomach, lung, breast, colorectum and cervix. An estimated 1 in 3 people will suffer from cancer during their lives [Cancer Research Campaign, Annual report, 1987].

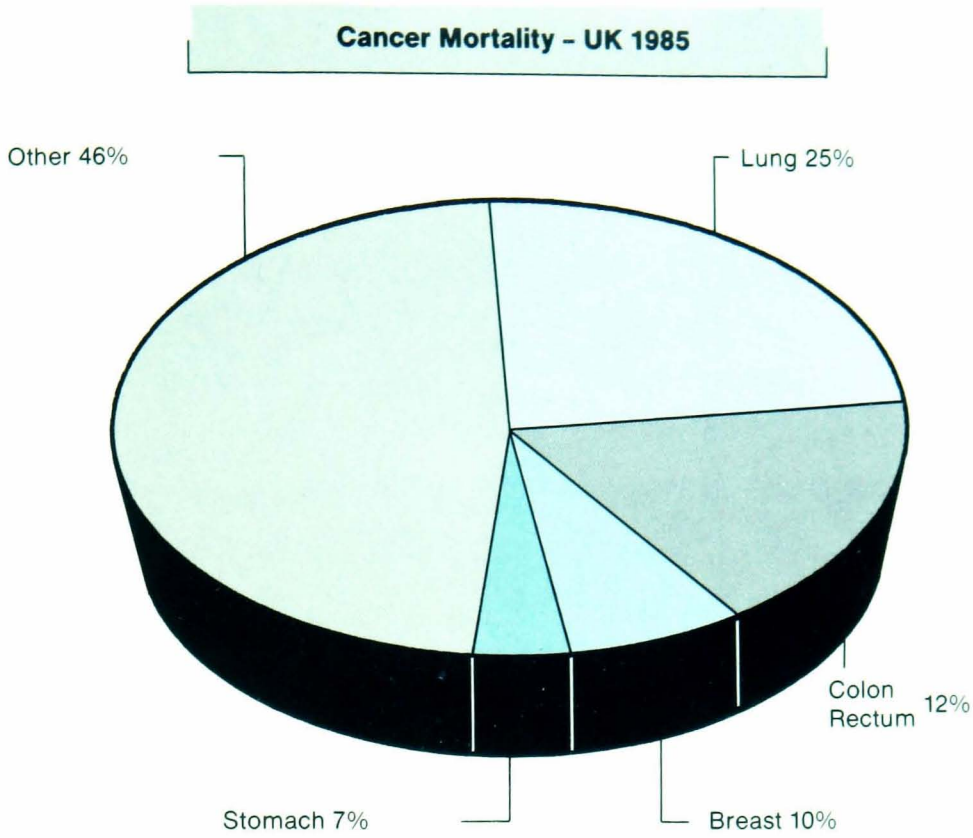
In the United Kingdom cancer deaths account for 25% of all deaths each year. Despite more than 70% of cancer deaths occurring in people over 65 years of age, cancer is the main cause of death in children and young adults after accidents and violence. The commonest cancers and the cancer mortality rate in the United Kingdom are shown in table 1.1.

Clearly cancer is a major problem, hence the need to fully understand its nature and origin, find new therapeutic approaches and refine and gain better understanding of existing ones. A major part of the studies in progress is the design, synthesis and screening of new antitumour agents. However, further success in this area relies to a large extent on a full understanding of the mechanism of action of antitumour agents used effectively in the clinic to date.

1.2 The nature of cancer

An understanding of the origin and nature of cancer is important before consideration of the therapeutic approach to treating this disease.

A malignant tumour or neoplasm consists of an abnormal and uncontrolled proliferation of cells which invades the surrounding tissues and can



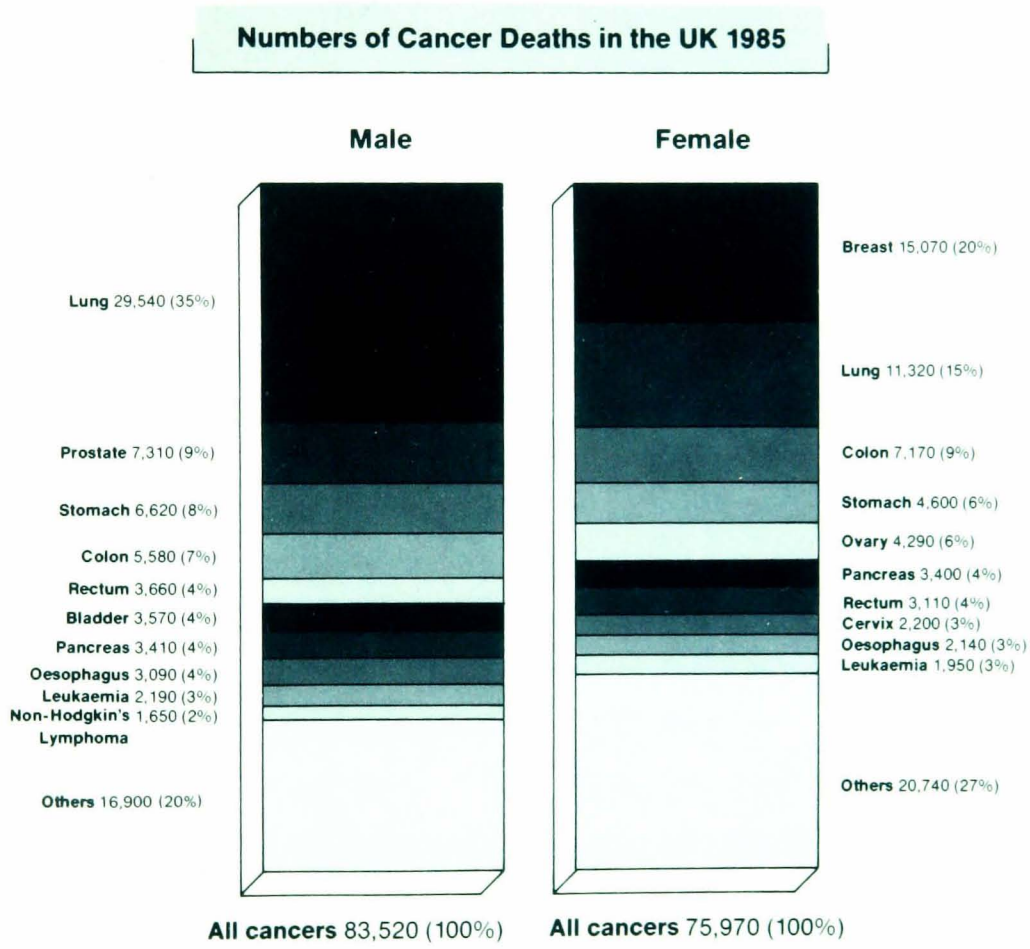


Table 1.1. *Cancer Incidence in the United Kingdom.*

(from Cancer Research Campaign factsheet 3.1, 1987)

metastasise to other parts of the body. Cancer has been ascribed to a 'malady' of genes arising from genetic damage such as recessive and dominant mutations, rearrangements of DNA and point mutations, leading to various distortions of gene expression or the biochemical function of gene products (Bishop, 1987). The theory that genetic damage might be responsible for cancer evolved from several lines of evidence (reviewed by Emery, 1985): 1) the detection of damaged chromosomes in cancer cells; 2) the susceptibility to cancer of cells unable to repair DNA; 3) the hereditary disposition of some people to cancer; 4) evidence that relates the mutagenic potential of substances to carcinogenicity; 5) the discovery that cellular genes (proto-oncogenes) in the form of oncogenes can cause neoplastic growth (reviewed by Nishimura and Sekiya, 1987).

1.2.1 *Genetic factors and cancer*

Cancers can be grouped into four categories with respect to genetic factors (reviewed by Emery, 1985):-

1) Rare genetic forms of cancer such as Xeroderma pigmentosum, and the increased frequency of acute leukaemia associated with Down's syndrome.
2) Common cancers which are inherited in an autosomal dominant trait in some rare families. The same type of cancer occurs over several generations of a family eg ovarian cancer.
3) The 'cancer family' syndrome, where a predisposition to cancer is inherited in an autosomal dominant trait.
4) The common cancers which are the largest group of cancers. These have a multifactorial basis possibly involving a genetic predisposition to cancer and exposure to an environmental carcinogen. However, there is no major inheritable component to these cancers.

The seminal discovery in this area was that made by Rous in 1910, who found that a cell free filtrate from chicken sarcoma could induce the same tumour in other chickens. Many viruses are now known to cause tumours including hepatitis B virus [liver cancer], Epstein Barr virus [Burkitt's lymphoma] and human papillomavirus [cancer of the cervix]. The events which occur when a virus infects a cell are shown in figure 1.1. Some viruses are able to transform the infected cell into a tumour cell. These viruses carry transforming genes or oncogenes (reviewed by Emery, 1984; Aaronson *et al.*, 1985; Nishimura and Sekiya, 1987) which code for a protein which mediates this transformation. The use of viral oncogenic gene probes from animal retroviruses has shown that human transforming sequences are homologous to various viral oncogenes [v-onc] and have been referred to as cellular oncogenes [c-onc]. It is believed that retroviral oncogenes originated from cellular genes or proto-oncogenes which were picked up at some point in their sojourn as proviruses. Many cellular oncogenes have now been identified in humans. Activated oncogenes have been found in a variety of malignant tumours including bladder, breast, lung colon and pancreas [all c-H-ras], Burkitt's lymphoma [c-myc] and neuroblastomas and sarcomas [c-N-ras] and chronic myeloid leukaemia [c-abl].

An important question is how do cellular oncogenes become activated to produce cancers? Several proposals exist to answer this question (reviewed by Bishop, 1987; Emery, 1985):-

1) A point mutation in a cellular oncogene which interferes with its expression. eg c-H-ras in bladder cancer (Reddy *et al.*, 1982).
2) A cellular oncogene may become activated by a relevant viral promoter when the virus becomes integrated into the DNA adjacent to the oncogene. eg T cell leukaemia (Gelmann *et al.*, 1984).
3) A chromosomal rearrangement involving translocation of a cellular oncogene to an adjacent site to a promoter or enhancer sequence, eg Burkitt's lymphoma and myeloid leukaemia (Rowley, 1983).
4) Induction of a point mutation in a cellular oncogene by a chemical carcinogen (Eva and Aaronson, 1983).

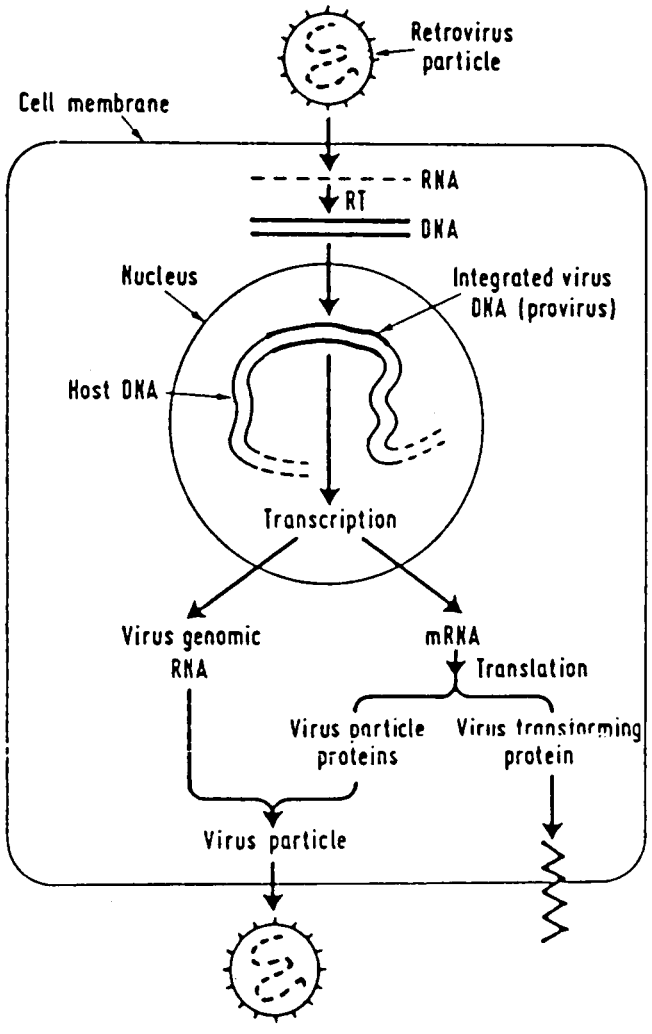


Figure 1.1. *Sequence of events which occur when a retrovirus infects a cell* [RT=reverse transcriptase]. (Emery, 1985).

The protein products of oncogenes have not yet been fully identified, however several oncogenes have been found to produce products similar to growth factors and growth factor receptors [reviewed by Aaronson *et al.*, 1985; Nishimura and Sekiya, 1987). For example platelet derived growth factor has been found to be structurally related to $P28^{sis}$ protein of Simian sarcoma virus (Hunter, 1984; Waterfield *et al.*, 1983)

It can be seen from section 1.2 that DNA is the critical molecule involved in the initiation of cancers. Before considering the treatment of cancer it is necessary to consider some important aspects of the structure and function of DNA.

1.3 Deoxyribonucleic acid

The primary structure of the deoxyribonucleic acid [DNA] duplex is shown in figure 1.2 (reviewed by Alberts *et al.*, 1983). The natural form of DNA is the B form in which the double helix is coiled in a right-handed direction, the chains turning to the right as they move upwards. However, local regions of the DNA molecule can have a left-handed configuration, known as Z form DNA because of the zig-zag path of the sugar-phosphate backbone. Another form of DNA is the A form which occurs due to puckering of the sugar backbone.

1.3.1 *Higher order packing of DNA*

In order to accommodate the long DNA molecules into the nucleus, a higher order structure of DNA exists. A series of DNA binding proteins exist, which mediate processes such as RNA synthesis, gene expression and act as structural proteins. These proteins are divided into histone and non-histone proteins. The non-histones are a multitude of proteins which have a wide range of functions, whilst the histones are primarily structural. When DNA is associated with histones it is known as chromatin. Four different orders of packing can be identified in chromatin (see figure 1.3)[reviewed by Alberts *et al.*, 1983; Nelson *et*

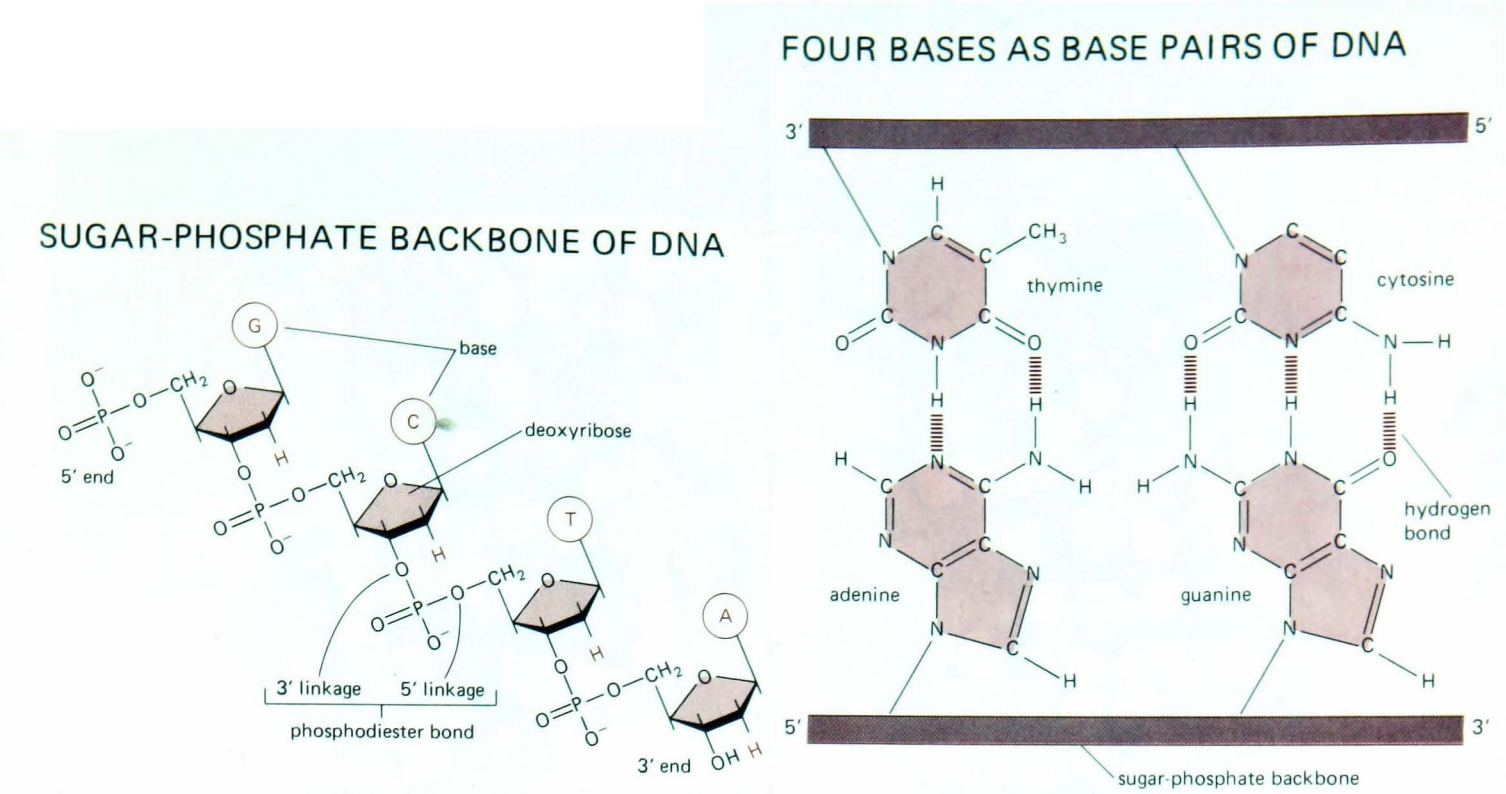


Figure 1.2. *The structure of Deoxyribonucleic acid.*

[Alberts *et al.*, 1983]

al., 1986):-

1. Primary coiling involves the supercoiling or further twisting of the DNA duplex in the direction of turn of the DNA strands.
2. Secondary coiling involves the coiling of the DNA duplex around a core of histone proteins (Composed of an octamer of histone proteins, two each of H2A, H2B, H3 and H4). Known as the nucleosome this repeating subunit with 166 to 168 base pairs (bp) of DNA wrapped in two left-handed superhelical turns around each histone core is separated by a region of linker DNA (variable up to 70bp long) giving it a 'beads on a string' structure.
3. Tertiary DNA folding involves the arrangement of the nucleosome into a higher order structure, the 30nm chromatin fibre or solenoid [cylindrical in shape]. This consists of either a helix with 6 nucleosomes per turn or a superbead configuration. Histone H1 is responsible for arranging DNA into this structure, lying outside the nucleosome, the central globular portion of this molecule spans the points of entry and exit of the DNA coil. It protects 15 bp on either end of the core associated coils but not the linker DNA. Removal of histone H_1 causes the nucleosome to separate.
4. Finally, quarternary DNA folding involves the folding of the chromosomes into looped domains. Eighteen of these loops form a radial array around the core of the chromosome to produce a miniband. The continuous formation of these radial loops produces a stack of minibands forming a metaphase chromatid.

DNA folding with relation to function is described in more detail by Alberts *et al.*, (1983), Nelson *et al.*, (1986), Eisenberg, (1987) and Yaniv *et al.*, (1986).

1.3.2 *Function of DNA*

Detailed accounts of the replication, transcription and translation of DNA into protein are given by Alberts *et al.* (1983). The way DNA is organised and its physical relationship with nuclear protein is highly significant in determining the state of the cell. The organisation of

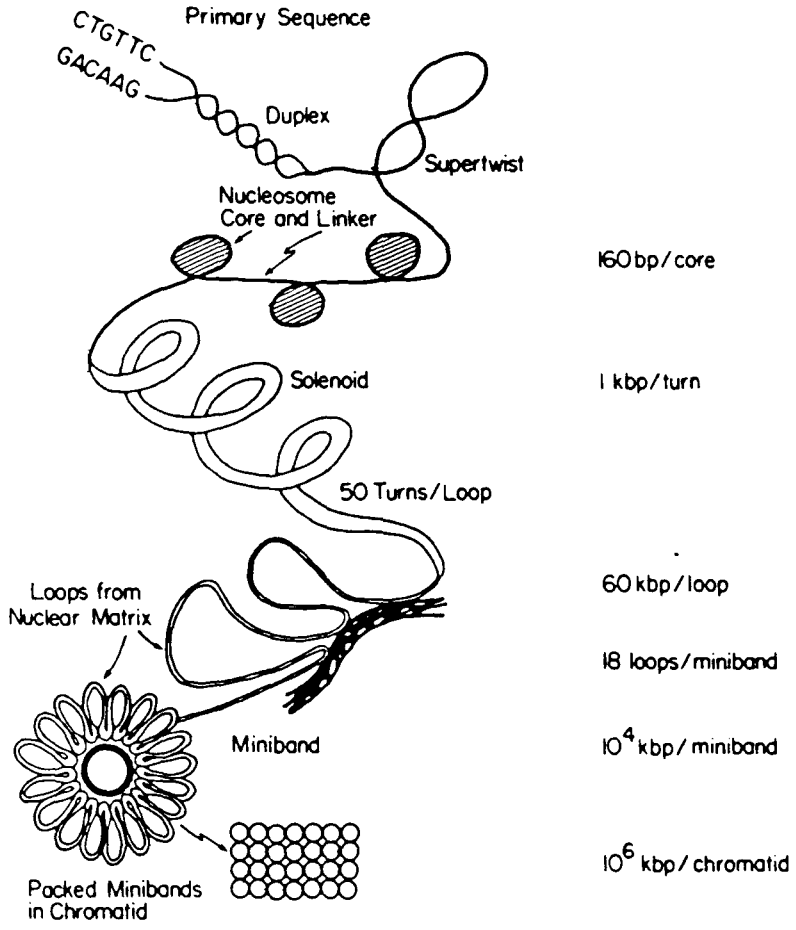


Figure 1.3. *Schematic diagram of the organisation of DNA within the chromosome.*

(Bohr *et al* 1987)

DNA into chromatin means that the conformation of active genes within this higher order structure must be modified so as to be accessible to transcription. This change is thought to be dependent on nuclear proteins, the structure of the nuclear matrix, methylation of DNA and other changes in the association between histones and DNA which are still to be elucidated.

1.4 The control of gene expression

The individual cells of an organism are distinct both in morphology and function. Highly specialised cells synthesise large amounts of one or several specific proteins. This differentiation of cell structure and function depends on changes in the selection of DNA sequences for decoding into proteins without irreversible changes to the DNA sequences themselves. Three classes of gene activity are recognised as being required for determining the characteristics of a particular cell: activation of housekeeping genes for general cell metabolism; activation of genes for 'luxury' proteins which typify a cell type eg muscle actin and myosin; hormone induced gene products. The regulation of gene expression may be exercised at several different levels [see figure 1.4]. One of the most important areas of gene regulation has been found to be at the transcriptional level (reviewed by Darnell, 1982). All gene regulation in prokaryotes is known to occur at the transcriptional level. There are considerable differences between the functional details of prokaryote gene control systems but the 'operon model' (reviewed by Sang, 1984) outlines the general principles of these systems.

1.4.1 *The operon model*

This model is based on the existence of a regulatory gene which codes for a regulatory protein which 1) interacts with a specific DNA sequence or operator which is located adjacent to the polymerase binding site [promoter] of a gene or several genes and 2) has its affinity for the

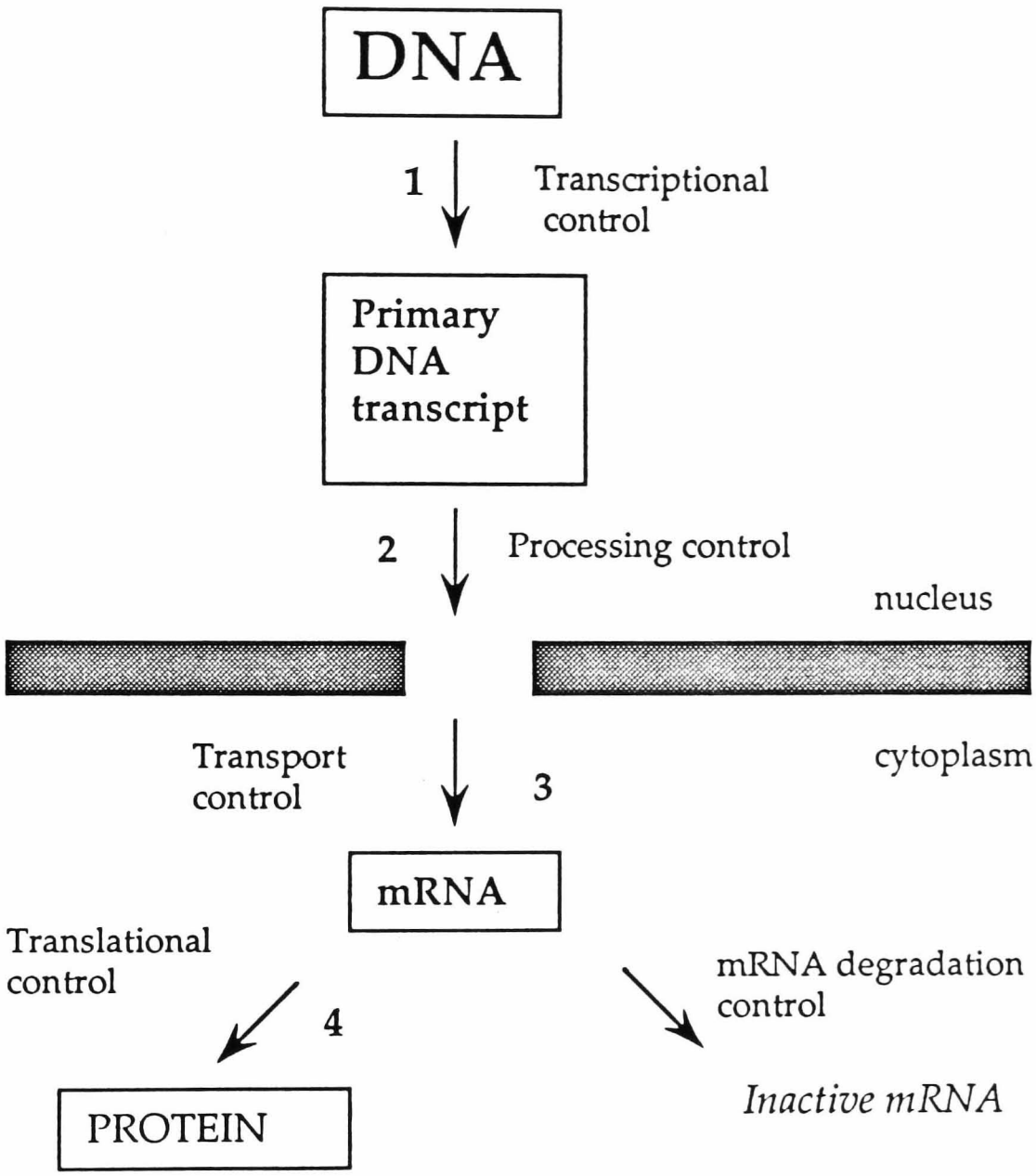


Figure 1.4 *.The different levels at which gene expression may be controlled in eukaryotes*

1: see text, **2**: reviewed by Darnell (1982), Mattaj (1984)
3: reviewed by Robertis (1983),

operator modified by a signaling ligand. The signaling ligand binds to the protein by weak secondary bonds [hydrogen bonds, ionic interactions and van der Waals forces]. Two types of control system are possible, a positive system where the regulatory gene product switches on the gene when combined with the signaling ligand and a negative system where this combination switches off the gene. These systems are outlined in figure 1.5 and table 1.2.

There is indirect evidence for regulatory proteins in eukaryotes acting as positive and negative regulators of gene expression, however in a typical eukaryotic cell only 7% of the total DNA sequences are ever transcribed, hence thousands of repressor proteins would be required for the remaining 93%. Hence it is believed that gene repression is controlled differently in eukaryotes, with gene regulatory proteins acting as gene activators (Alberts *et al.*, 1983).

1.4.2 *The Britten and Davison model of gene expression (1969).*

This model (reviewed by Sang, 1984) [figure 1.6] assumed that:-

a) There are effector substances which are signals external to the genome which interact with genome sensors.
b) The sensor is a sequence of DNA and a specific sensor protein which specifically binds its appropriate effector.
c) Effector-sensor interaction results in transcription of an adjacent integrator gene set which codes for sequence specific activator proteins.
d) These proteins recognise receptor sequences and the associated gene or genes are transcribed.
e) Unlike the operon model a particular receptor sequence is not unique to a particular gene set. There maybe more than one receptor sequence associated with a gene or gene set, thus an activator may switch on more than one gene and one gene may be switched on by more than one activator.
f) The integrator set determine the activator messages, which may not be exclusive as the same message may be repeated in different

Effect of regulatory gene product	Positive control Apo-inducer switches on the operon	Negative control Apo-repressor switches off the operon
Inducible systems		
Co-activator function	Co-inducer activates apo-inducer	Co-inducer inactivates apo-repressor
Regulatory gene deletion	Uninducible	Constitutive
Regulatory gene mutation*	Constitutive	Uninducible
Operator deletion	Uninducible	Constitutive
Repressible systems		
Co-activator function	Co-repressor inactivates apo-inducer	Co-repressor activates apo-repressor
Regulatory gene deletion	Super-repressed	Derepressed
Regulatory gene mutation*	Derepressed	Super-repressed
Operator deletion	Super-repressed	Derepressed

*The mutation prevents binding with the co-activator, but not with the operator. The presence of a normal regulatory gene cannot interfere with this so the mutant effect is dominant.

Table 1.2. *Prokaryote control systems.*

(Sang, 1984)

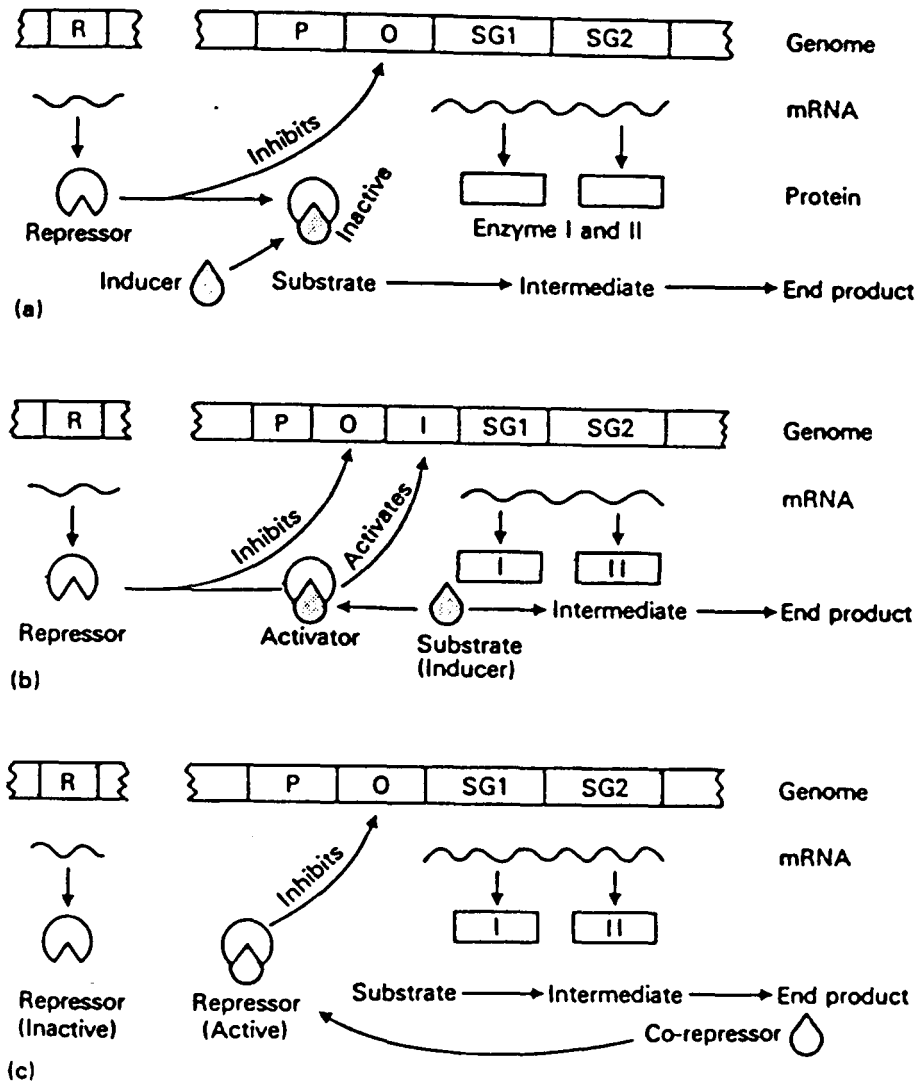


Figure 1.5. *Operon organisation.*

(a) Negatively controlled inducible system in which the regulatory product (R) combines with the operator (O) to prevent transcription of the two structural genes (SG1 and SGII), but is inactivated by the inducer thus permitting transcription. (b). Positively controlled inducible system, in which the operon is activated by the regulatory gene product (apo-inducer) combining with the substrate (co-inducer) and acting at the initiator sequence to initiate transcription. (c). Negatively controlled repressible system, where the regulatory gene product is inactive until combined with the co-repressor (usually end product) which inhibits the operator. P, promoter sequence; I, initiator sequence. Table 1.2 summarises the details. (Sang, 1984).

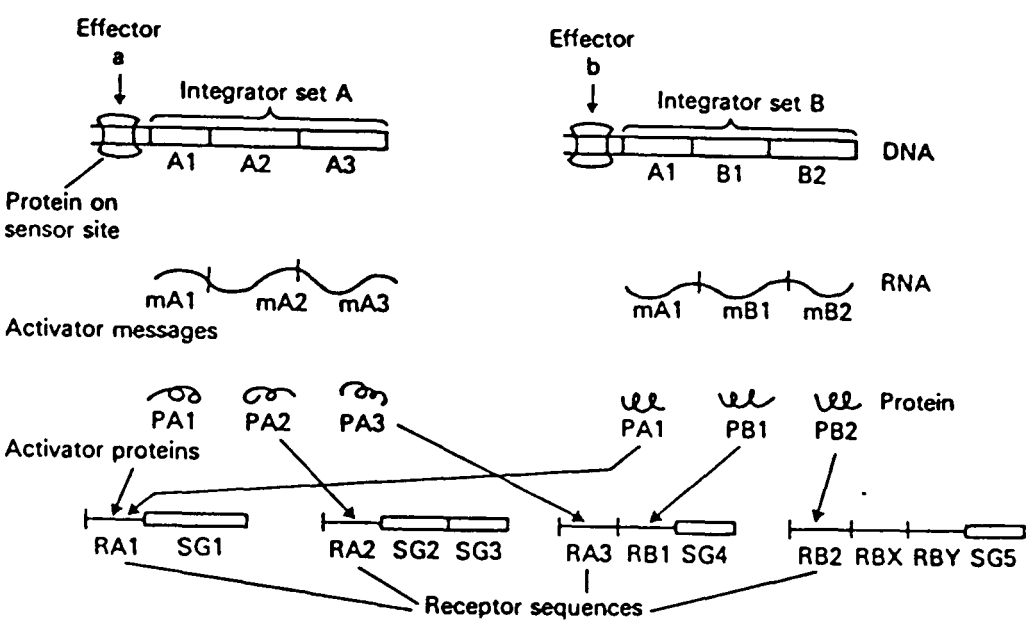


Figure 1.6. *The Britten and Davidson model.*

For simplicity only two integrator gene sets are illustrated. Each is activated by its specific effector a or b which combine specifically with the proteins of their sensor sites. This causes the coordinate transcription of the entire integrator gene set, and the formation of the activator mRNA (mA1 etc.) and of the activator proteins (pA1 etc.). These activator proteins (or mRNAs) combine with specific receptor (RA1 etc.) sites (operators) adjacent to structural genes (SG1 etc.), which are then transcribed and translated. (Sang, 1984).

integrator sets.

g) The activator message set (RNA) may be involved with the selective transcription of genes without being translated into protein. This allows the activation process to be intranuclear and independent of protein synthesis.

However this model was later modified as it assumed that the most important control of gene expression was transcriptional.

1.4.3 *The Davidson and Britten model of gene expression (1979)*

Like the genome most nuclear RNA [nRNA] contains interspersed repeated sequences. Regulated transcription of these sequences (for example Darnell, 1982; Konarska *et al.* 1985; Mattaj, 1984) has been found to have a key role as opposed to structural gene transcription as in the previous model. An interaction between the structural gene nRNA's and the transcriptionally-regulated nRNA's is assumed since the structural gene mRNA's are selectively processed.

The constitutive gene transcript [CT] carries the coding sequence flanked by short repetitive sequences, accounting for the complexity of this mRNA class in the nucleus. The integrating transcription unit [IRTU] is preceded by a nucleoprotein sensor which when activated by an external signal, results in selective transcription of the unit in a cell specific fashion. The IRTU carries interspersed repetitive and single copy sequences. The IRTU transcript [IRT] is the cell specific nRNA which can combine only with the gene transcripts carrying complementary , matching repeated sequence. Only this CT-IRT complex can be processed. Since the repetitive sequences of an IRT may be matched by repetitive sequences of a number of CT's the IRT will control the processing of a battery of CT's as in the previous model with the integrator gene sets. The gene battery of that model now becomes the set of genes bearing the same repeated sequences. This model is illustrated in figure 1.7.

The above models (reviewed by Sang, 1984) are concerned with the

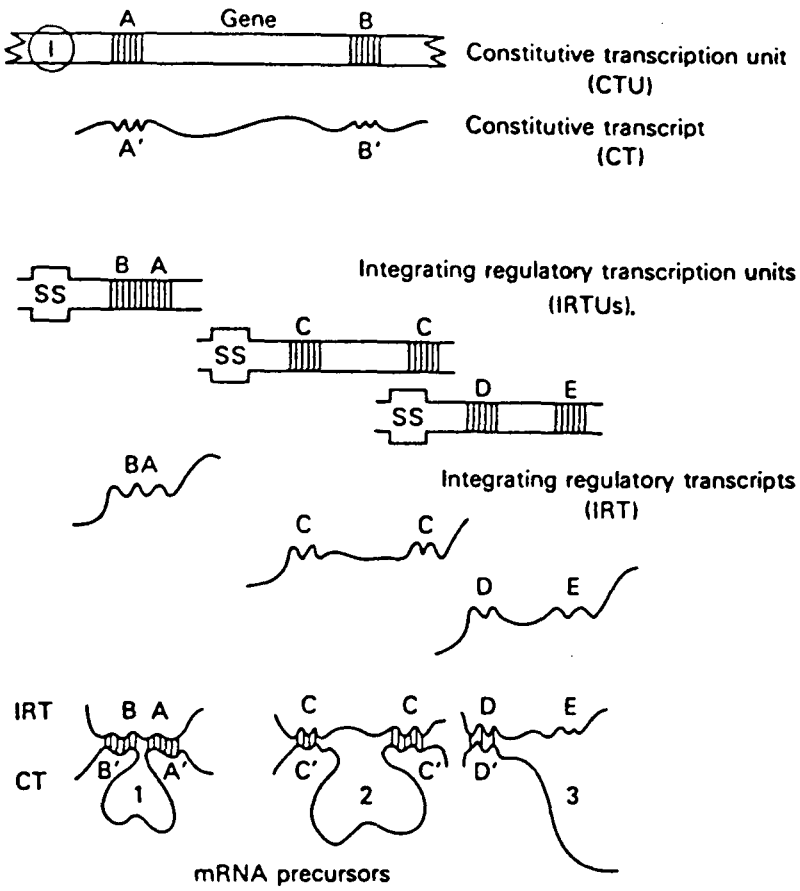


Figure 1.7. *The Davidson and Britten model.*

The CTU contains the structural gene, its introns and flanking sequence, including the initiation site (I) and the repetitive sequences A and B. This is constitutively transcribed into the CT (nRNA). Three possible forms of of IRTUs are shown, each with different arrangements of interspersed repetitive sequences (A,B,C,D and E). Their transcription is controlled by nucleoprotein sensors (SS) which respond to external signals and then make the IRT(nRNA). The IRTs duplex with the CTs, by sequence dependent base pairing between the repeated elements. Three possible arrangements are shown. These IRT-CT complexes must form before the mRNA can be processed and then translated. (Sang, 1984).

differential regulation of transcription and assume there is no specific role in the organisation of chromatin in this respect. The opposite view allocates a regulatory function to the chromatin proteins including non-histone proteins. The packaging of DNA into chromatin is a feature unique to eukaryotes which is important in gene regulation. DNA must be transcribed whilst it is bound to the nucleosome, however most chromatin is highly condensed and inaccessible to polymerases so it must be decondensed to allow transcription to take place. Transcribable DNA regions have been found to be accessible to digestion by the endonuclease DNase I indicating that it is the state of chromatin which determines the accessibility of this enzyme. Histone H_1 is found to be bound less tightly to some transcribed regions and histones in active nucleosomes are unusually highly acetylated. The small non-histone proteins HMG14 and HMG17 have been found to be bound to active nucleosomal genes [reviewed by Sang, 1984). In addition, topoisomerase enzymes [see section 1.7] have been found to be intimately involved in mediating the topology of DNA during transcription. All of these factors may play an important role in uncoiling the chromatin of active genes (reviewed by Alberts *et al.*, 1983). It is thought that eukaryotic gene activation occurs in two stages, firstly the binding of a gene regulatory protein to a specific DNA sequence and secondly, this causes a structural change in the neighbouring chromatin which is propagated for the length of the gene or the entire length of a chromosome looped domain (reviewed by Alberts *et al.*, 1983).

The control of gene activation has been described at the molecular level. At the cellular level, the growth and division of cells is controlled by the cell cycle. It is the events of this cycle and its relationship to the growth of normal and tumour tissue which determine the effectiveness of antitumour agents. Before specifically describing the interactions of antitumour agents with DNA it is necessary to describe these relationships.

1.5 Chemotherapy and the cell cycle

1.5.1 *The cell cycle*

The cell cycle (reviewed by LLoyd *et al.*, 1987; Alberts *et al.*, 1984) is the period between one mitosis and the next one in either of the daughter cells. This period is divided into four phases as shown in figure 1.8. G_1 phase is the period between mitosis and the onset of DNA synthesis, S phase the period of DNA synthesis, G_2 phase the period between completion of DNA synthesis and mitosis and M phase is the period of mitosis (or nuclear division) and cytokinesis (or cytoplasmic division). Within G_1 there is a restriction point (R). When this is reached the cell is committed to divide. Cells in any given population are divided into three categories with respect to the cell cycle:-

1. Continuously dividing or cycling cells.
2. Resting or non-cycling cells which can be induced to start dividing with an appropriate stimulus.
3. Cells that have left the cell cycle and will die without dividing again.

Resting cells are termed as being in G_0, a term which has been derived to acount for the variability of G_1 phase of cultured cells. This variability in time span of G_1 is the major determinant of the cycle time of a cell population. There are two main theories for how cells transit from G_1 phase to S phase (reviewed by Lloyd *et al.*, 1987):-

1. It has been proposed that a critical concentration of a 'trigger protein' is required in order for a cell to progress from G_1 to S phase.
2. The transition probabilty model proposes that the cell cycle is divided into two states:- the B state [containing S, G_2, M AND G_1) in which cells are proceeding towards division and the A state [contained in G_1] where the cells are awaiting a random triggering event before proceeding. This model suggests a cell may remain in

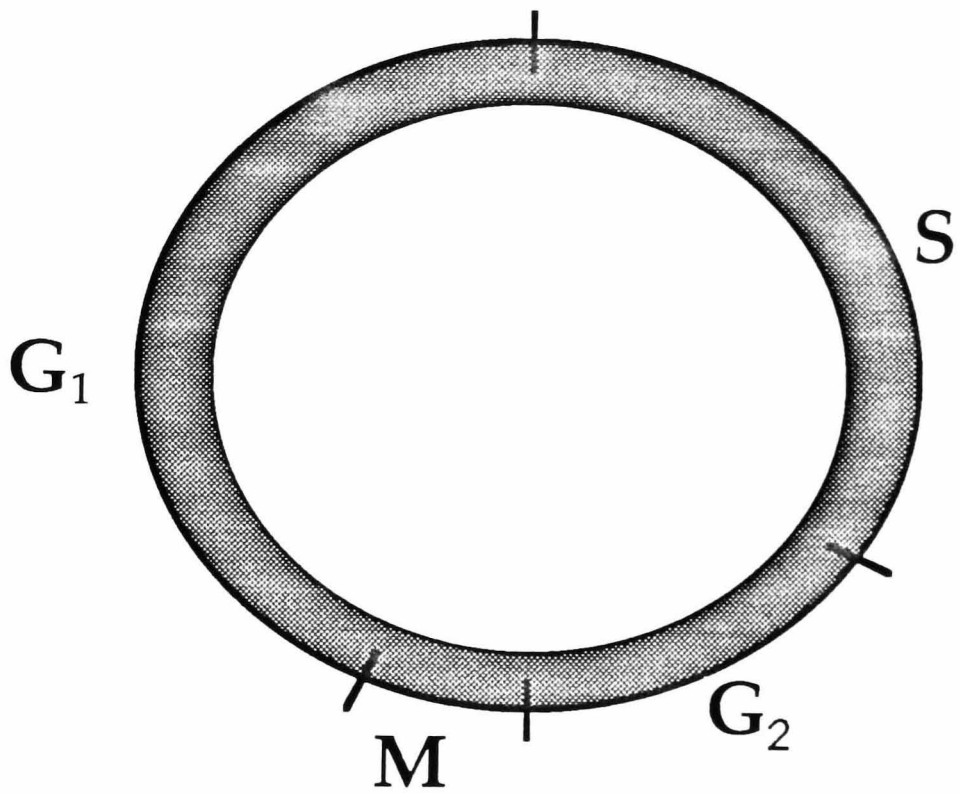


Figure 1.8. *The Cell Cycle*

the A state for any length of time, but in steady state conditions the probability of a cell leaving the A state and entering the B state is constant.

1.5.2 *Tissue growth kinetics*

1.5.2.1 *normal tissue*

In a similar manner to cells within a population, normal tissues can be divided into four categories (reviewed by Pardee, 1985):-

1. Cell renewal tissues where there is extensive birth and death of cells eg. mucosa of bladder and skin.
2. Non-proliferating tissues such as nonstriated muscle and neuronal tissue.
3. Cells with little tendency for proliferation in the steady state such as smooth muscle and capillary endothelium.
4. Cells that proliferate in response to physiological requirements (for example liver cells after hepatectomy).

The cells within a population are divided into compartments as shown in figure 1.9. The stem cells are the progenitors of the cell compartment. In the steady state they are resting and act as reserve cells. However, they give rise to the proliferation compartment when expansion and maturation is occurring. It is this compartment which is most susceptible to cytotoxic agents. Finally, the cells no longer divide and are incapable of cell division. These cells have a finite lifespan. As long as the mature compartment is equal to cell death the population of cells does not expand.

1.5.2.2 *Tumour tissue*

In tumour tissue the cell population is divided into similar

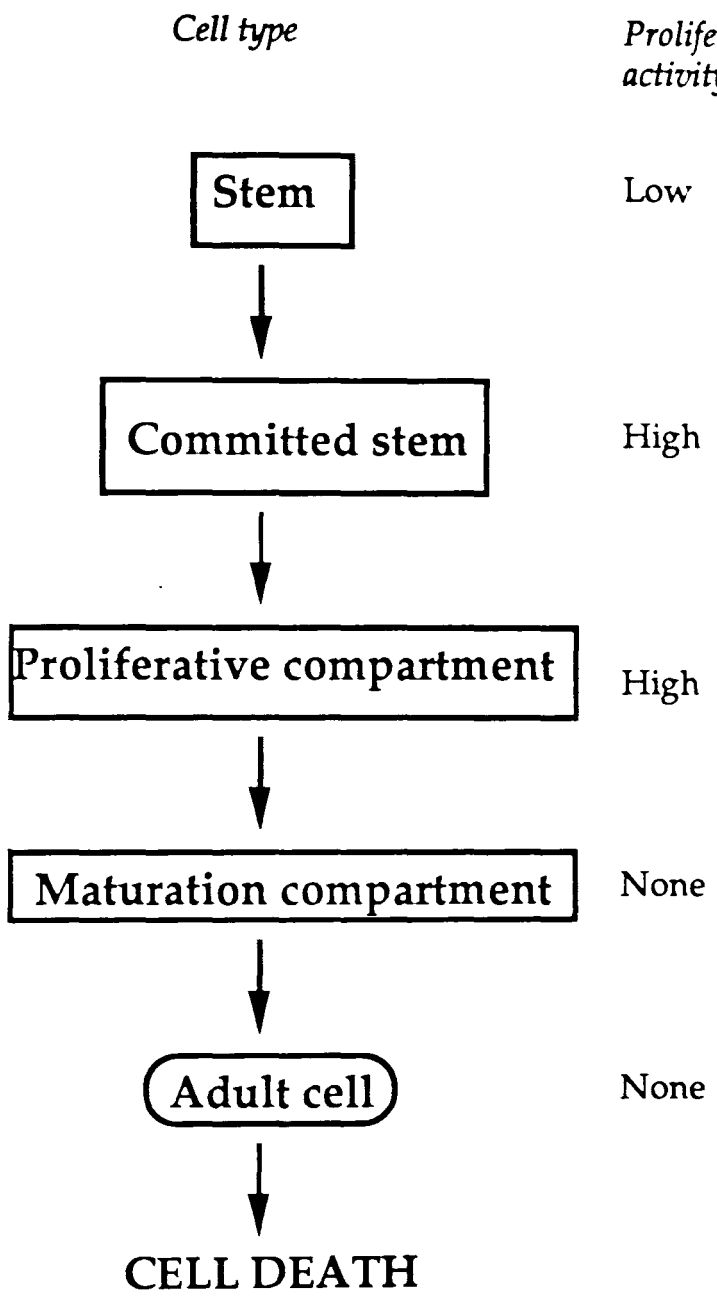


Figure 1.9. *Diagrammatic representation of the organisation of a cell renewal system.*

compartments. However, unlike normal tissue cell growth does not normally equal cell death, so a net increase in tumour size is the result (reviewed by Buick and Pollak, 1984; Hill, 1978; Pardee, 1985). In consequence tumour tissue has a higher 'growth fraction' [higher number of dividing cells]. This difference between normal and tumour cell populations is the basis of the differential effect of chemotherapy to the two tissue types. As in normal tissue, the stem cells are responsible for maintaining the integrity and continued survival of the cell population. The stem cells are capable of an infinite number of cell divisions, in contrast to other cell types. The malignant stem cells in a tumour mass are capable of self renewal and migration, allowing the primary tumour to grow as well as initiating metastases. Within the stem cell population there may be cycling cells which are sensitive to chemotherapeutic agents which affect proliferating cells, or resting cells which are insensitive to such agents. The state of the cells will depend on their accessibility to the capillary blood supply for oxygen and nutrients. Resting cells may re-enter the cell cycle to replace cells destroyed by therapy. Therefore in chemotherapy the stem cells must be regarded as the critical target. Successful therapy depends on differential activity of agents on malignant and normal cells.

Antitumour agents produce three distinct classes of survival curves when used in the treatment of normal and malignant cells:-

1. No difference in toxicity to normal and malignant cells, eg. X-irradiation, therefore termed as 'non-specific'.
2. More toxic to malignant than normal cells, cell kill being directly related to concentration until a plateau is reached. This is termed phase specific because the drug is killing cells at specific parts of the cell cycle. Resting cells are not affected.
3. Survival curves exponential with dose, no difference in slope being seen with normal and malignant cells. This is termed cycle specific because the drug kills proliferating and resting cells. However, dividing cells are more sensitive than resting cells throughout the cell cycle.

Thus specificity for tumour cells relies on the greater growth fraction

in this tissue. Examples of the classification and sites of action of antitumour agents is given in figure 1.10. Generally drugs can exert two main effects on cells which may or may not be expressed at the same point of the cell cycle:-

a) A lethal cell killing effect which is maximum at certain phases of the growth cycle. Generally occurs at high drug doses.

b) An effect which stops cell cycle progression by arresting cells at a specific phase. This is often reversible. This generally occurs at low doses of drug. By arresting the cell at a specific point a drug may inhibit its own lethal effect if this is expressed at a different phase.

It is quite clear from what has been described in this section that there is an intimate relationship between chemotherapy and cell division. The central event of cell division is the replication of DNA. This process is therefore a vulnerable target in cell populations which are continuously renewing or expanding such as tumours and some normal tissues. Indeed the majority of antitumour agents have been shown to interact with DNA, disrupting its structure, synthesis and function.

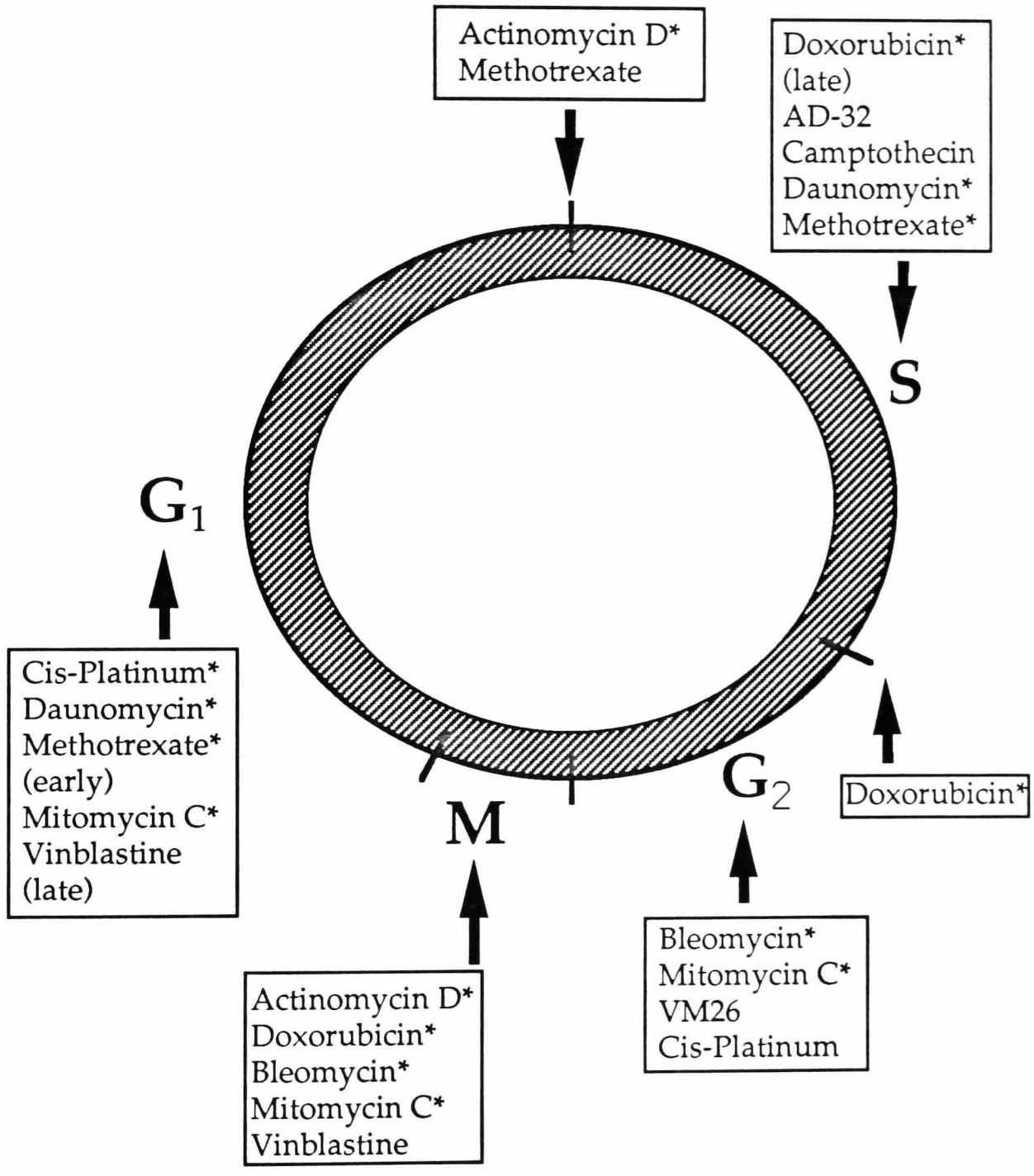


Figure 1.10. *The phases of the cell cycle where some antitumour agents exert their effects*

*indicates more than one site of drug action

1.6 The interaction of antitumour agents with DNA

There are several mechanisms by which antitumour agents have been shown to interact with DNA. The concept of DNA being a 'receptor' for antitumour agents has recently been reviewed by Hurley and Boyd (1988).

1.6.1 *Indirect effects on DNA*

The antimetabolites are agents that by virtue of structural similarity with physiological intermediates are accepted as imitation substrates for biochemical reactions which are vital for a particular cell process. An example is the antifolate methotrexate which exerts its effect through inhibition of the enzyme dihydrofolate reductase. This enzyme is responsible for maintaining the intracellular pool of reduced folates [tetrahydrofolates] which are the source of one carbon groups for synthesis of purine nucleotides and thymidylate. Therefore, the net effect of this drug is an inhibition of purine synthesis, hence a disruption of DNA synthesis (reviewed by Chabner and Myers, 1985).

1.6.2 *Binding of antitumour agents to DNA*

1.6.2.1 *Intercalative binding*

Reviews of DNA intercalation are given by Neidle and Abraham (1987). The basic postulate for intercalation is that the flat polycyclic aromatic ring system of a drug molecule can fit between the stacked base pairs of the double helix. There are three basic requirements for a drug to be considered as intercalated (Waring, 1981):-

a) The helix must be extended
b) The helix must be locally unwound by drug binding.
c) The plane of the aromatic chromophore of bound drug must be parallel

to that of the base pairs.

All these characteristics vary considerably for different drugs, the standard reference intercalator being ethidium (figure 1.11). As the amount of intercalator bound increases the negative (right handed) supercoiling of DNA is reduced, lost then reversed [followed by falling sedimention coefficient, viscosity or mobility on gels].

Antitumour agents that intercalate include the anthracycline antibiotics (Aubel-Sadron and Londos-Gagliardi, 1984) [see section 1.16.1], aminoacridines (Neidle and Abraham, 1987), quinoxaline antibiotics (eg Echinomycin), antitumour alkaloids (eg ellipticine) and other antitumour agents such as Actinomycin D [reviewed by Waring, 1981]. Bifunctional intercalative agents have also been developed in an attempt to increase binding affinity and also to attain more selective binding to preferred sites in DNA (eg diacridines, reviewed by Waring, 1981). However, the antitumour activity of these agents has not always been more effective than monomeric intercalators.

1.6.2.2 *Non-intercalative binding*

This category contains drugs which bind to DNA but not by a proven intercalative mechanism. These drugs bind mainly to the surface of the DNA molecule by electrostatic interactions. An example of this type of drug are Mithramycin antibiotics (Waring, 1981).

1.6.2.3 *Covalent binding*

Bifunctional alkylating agents including nitrogen mustards, nitrosoureas, triazenes and ethyleneimines form covalent bonds to nucleophilic centres, especially proteins and nucleic acids (reviewed by Chabner and Myers, 1985). Most alkylating agents form a carbonium ion in aqueous solution (Chabner and Myers, 1985). When the alkylating groups are chloroethyl groups [eg nitrogen mustard], a preliminary

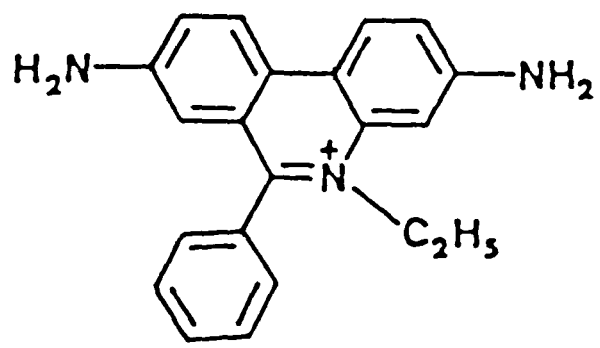


Figure 1.11 *The structure of ethidium.*

cyclisation to form an unstable imonium ion occurs, with spontaneous opening of the three membered ring to give the alkylating intermediate $R\text{-}CH_2\text{-}CH_2^+$. This charged group then attacks nucleophilic sites on nucleic acids, proteins and other small molecules. The N(7) position of guanine accounts for 90% of alkylated sites in DNA [Reviewed by Gianni *et al.*, 1983].

When two alkylating groups are situated on each drug molecule such as in nitrogen mustard which has two chloroethyl groups, DNA cross-linking occurs, which is thought to be the major effect of these agents [Chabner and Myers, 1985].

Other alkylating agents such as mitomycin C require activation by NADPH dependent enzyme systems before alkylation and crosslinking can occur (Hoey *et al.*, 1988; reviewed by Kennedy *et al.*, 1980) [see section 1.8.2]. Mitomycin has two reaction centres, one is the strained three membered aziridine ring and the other being the methylurethane side chain at position 10 [see figure 1.12]. Other alkylating agents include cyclophosphamide, Cisplatin (reviewed by Chabner and Myers, 1985), 2,5-bis(carboethoxyamino)-3,6-diaziridinyl-1,4-benzoquinone (AZQ) (figure 1.18) [reviewed by Gutierrez, 1989], and 2,5-bis(2-hydroxyethylamino)-3,6-diaziridinyl-1,4-benzoquinone (BZQ) [Butler *et al.*, 1989].

1.6.3 *Strand breaking interactions*

A common characteristic of many DNA interactive drugs is the production of DNA strand breaks. This includes the DNA intercalators, non-intercalators and alkylating agents. Single and double strand breakage of DNA by intercalating agents may arise due to attack by reactive drug intermediates or as a result of DNA repair activity or topoisomerase activity [see section 1.7] being disrupted by the presence of the drug. The number of single strand breaks caused by excision repair endonuclease activity is balanced by the resealing of single strand breaks by polynucleotide ligase [reviewed by Alberts *et al.*, 1983;

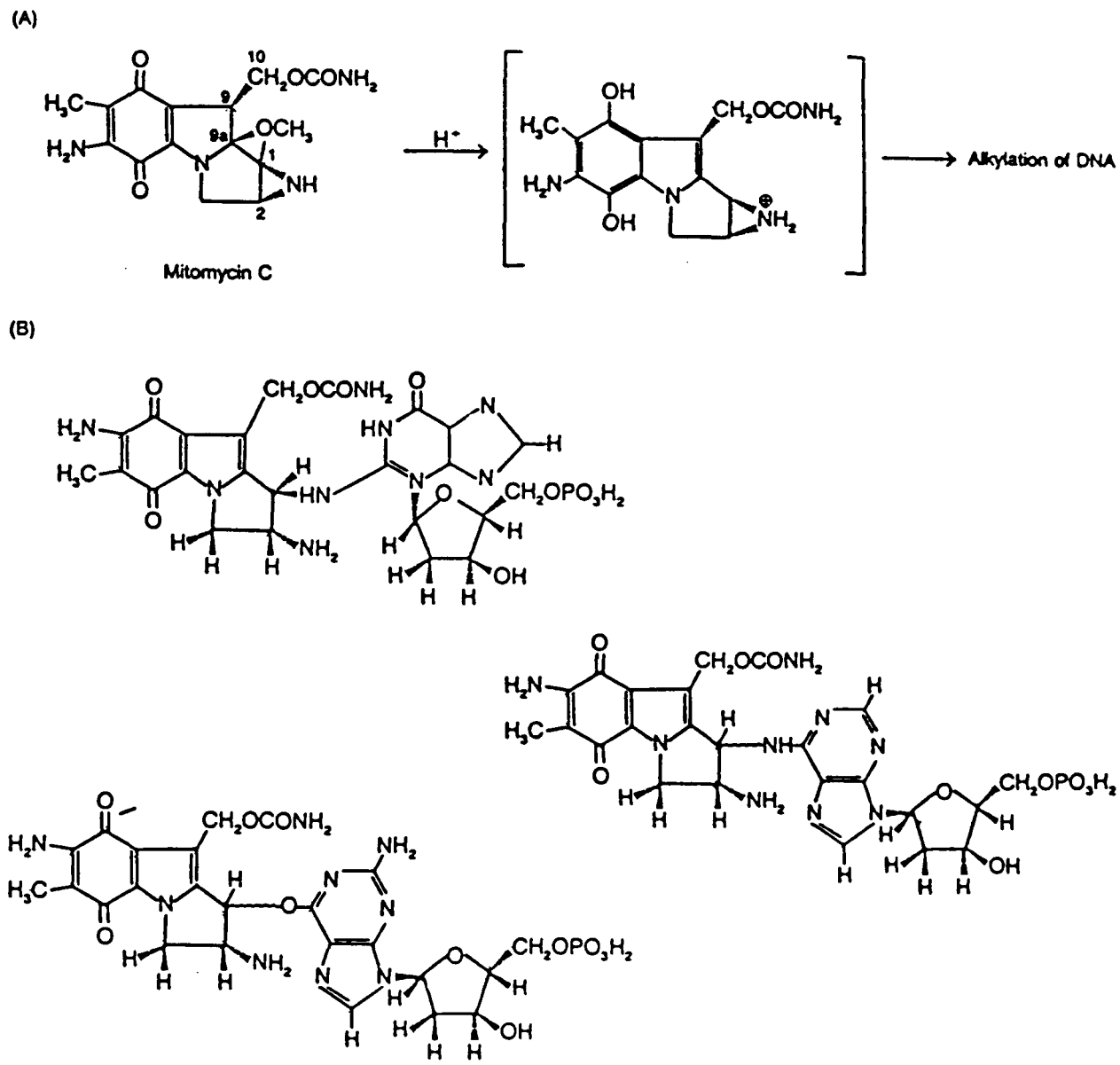


Figure 1.12 *Alkylation of DNA by mitomycin C.*

Three kinds of modified nucleotides isolated from DNA alkylated with reductively activated mitomycin C are shown.

Friedberg, 1987]. DNA repair synthesis is required for this process. Consequently, an inhibition of any part of this process may lead to an increase in strand breakage. The nature and causes of DNA strand breakage produced by drug free radical intermediates is considered in greater detail in section 1.9.2. Several types of DNA damage are specifically seen with alkylating agents:-

1.6.3.1 *Alkali-labile sites*

These are proposed to be an indicator of genetically significant DNA damage caused by alkylating agents, which react with DNA at multiple sites (reviewed by Kohn, 1981 and 1983). The guanine O_6 position, a major site of nucleophilic damage is a relatively weak nucleophile which reacts only with potent nucleophiles. O_6-alkyl-guanine residues are lesions that are repaired by alkyl transferase mechanisms which do not involve strand breaks as an intermediate [D'Incalci *et al.*, 1988). Such weak nucleophilic sites are converted to strand breaks in the presence of an alkaline solution. In addition, alkylated DNA phosphate groups tend to form strand breaks in alkali. These strand breaks can be measured by the alkaline elution technique [see section 1.11.1].

Alkylation sites at guanine N_7, a strong nucleophilic site are repaired by glycosylase and alkaline phosphatase-endonuclease reactions which do involve strand breaks so drug induced strand breakage may occur via this mechanism.

1.6.3.2 *Interstrand cross links*

Agents that bind to DNA at two sites are much more cytotoxic than monofunctional agents. Most bifunctional agents produce inter and intra-strand crosslinks and DNA-protein crosslinks between DNA and nuclear proteins which can block DNA and RNA synthesis eg mitomycin C (reviewed by Kennedy *et al.*, 1980), bis-platinum(II) complexes, AZQ

[reviewed by Gutierrez, 1989; King *et al.*, 1984; Szigiero and Kohn, 1984] and BZQ (Butler *et al.*, 1989).

1.7 Interaction of antitumour agents with nuclear enzymes

Within the nuclear matrix there are a large number of enzymes which are intimately involved in the management of DNA and its functions, including topology, replication, transcription and repair. In recent years it has been found that certain antitumour agents possibly exert their cytotoxicity by specifically interfering with some of these enzymes. The main focus of attention has been the DNA topoisomerases (reviewed by Drlica and Franco, 1988).

1.7.1 *Interaction of antitumour drugs with topoisomerases*

Reviews on this subject are given by Lock and Ross (1987), Ross (1985) and Bodley and Liu (1988). DNA topoisomerases are enzymes that alter the topological state of DNA. Cellular processes such as replication, transcription and recombination require these topological changes in the structure of DNA (reviewed by Wang, 1985). Topoisomerase II has recently been reported to act specifically during mitosis (Holm *et al.*, 1989). Thus, the topoisomerases are essential in cell growth processes. Topoisomerases catalyse DNA relaxation/supercoiling, catenation/ decatenation and knotting/ unknotting [see figures 1.13 and 1.14] (Maxwell and Gellert, 1986).

The topological state of closed-circular duplex DNA that is not catenated or knotted is characterised by its linking number, which expresses the number of times the two strands are interwound. There are two types of topoisomerases, defined by their mechanism of action with respect to the changes in linking number they confer.

1.7.2 *Type II topoisomerases*

These break both strands of the DNA helix, with a four base stagger

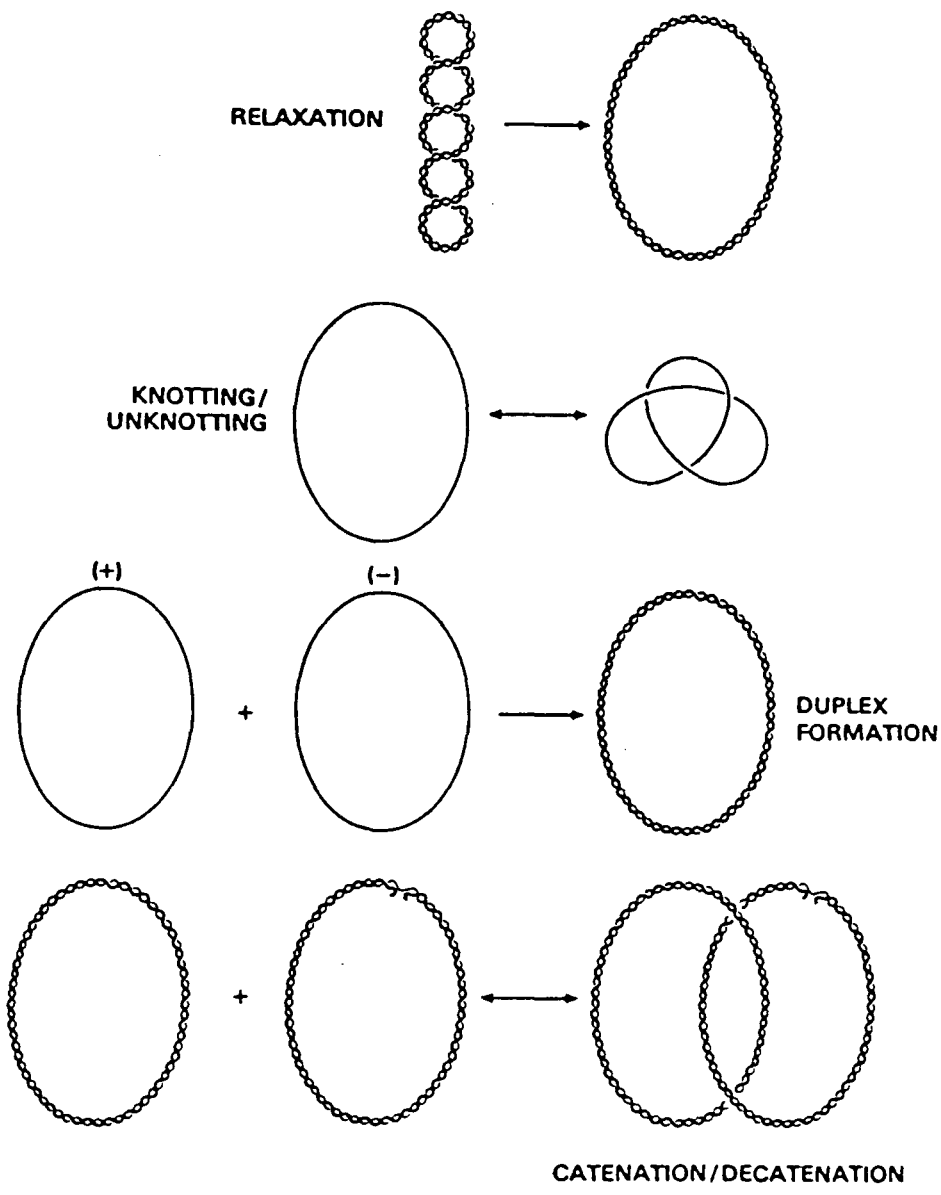


Figure 1.13. *Reactions of type I topoisomerases*

Note the catenation or decatenation of circular duplexes will occur only if one DNA molecule bears a single strand break (Maxwell and Gellert, 1986)

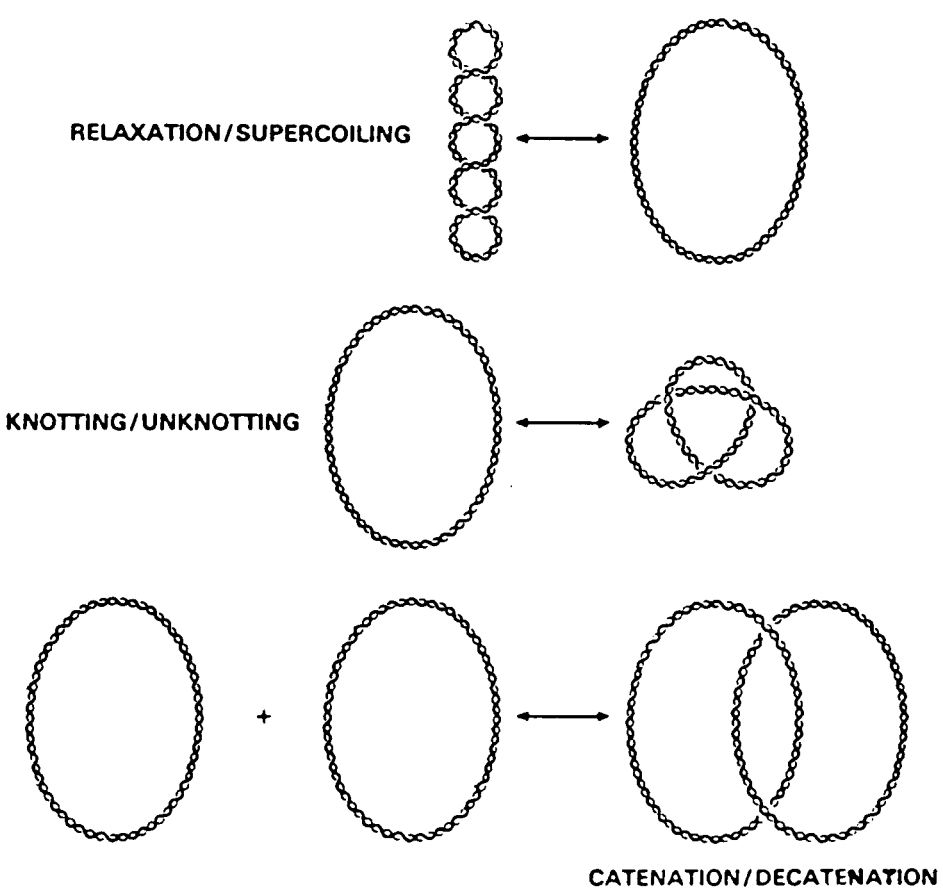


Figure 1.14. *The reactions of type II topoisomerases.*

(Maxwell and Gellert, 1986)

between the cuts, allowing a second duplex to pass through the break before it is resealed. The linking number of the DNA is therefore changed by two. Eukaryotic topoisomerase II [reviewed by Zwelling, 1985; Wang, 1985; Glisson and Ross, 1987] has a molecular mass of 340,000 Da, having two 170,000 Da subunits. The hydrolysis of ATP is an integral part of its action.

The addition of a strong protein denaturant such as sodium dodecyl sulphate [SDS] to a reaction mixture of topoisomerase II, duplex DNA and some antitumour agents produces single and double strand breaks in the DNA, with a subunit of topoisomerase covalently attached to each 5′ phosphoryl end of the break (see figure 1.15). The double strand break is a four base pair staggered cut with recessed 3′ hydroxyl ends. It has been proposed that the covalent DNA-protein adduct is derived from an intermediate of the topoisomerase reaction, the 'cleavable complex'. This complex consists of DNA and enzyme in a conformation which is cleaved or susceptible to cleavage

The observation that there were similarities between the cleavable complex formation and DNA protein crosslinks caused by some antitumour agents led to investigation of the role of topoisomerases in the mechanism of action of these drugs (Zwelling *et al.*, 1981). Studies with alkaline elution [see section 1.11.1] showed that DNA damage induced by intercalative agents (Tewey *et al.*, 1984) such as doxorubicin (Tewey *et al.*, 1982) , and 4′-(9-acridinylamino)methanesulfon-m-anisidide [m-AMSA](Nelson *et al.*, 1984) was associated with formation of protein linked strand breaks. Similar effects were seen with non-intercalative drugs such as etoposide, (Chen *et al.*, 1984). However, it has recently been found that these drugs, including etoposide, teniposide and the N-acyl anthracyclines AD32 and AD41, do bind to DNA (Chow *et al.*, 1988). All these agents interfered with the breaking and rejoining reaction of topoisomerase II by trapping an abortive enzyme-DNA 'cleavable complex' [see figure 1.15]. When this complex was treated with protein denaturants such as SDS and proteinase K DNA,strand breaks were revealed [single (Muller *et al.*, 1988) and double (Wang, 1985)]. These drugs are effectively converting the enzyme

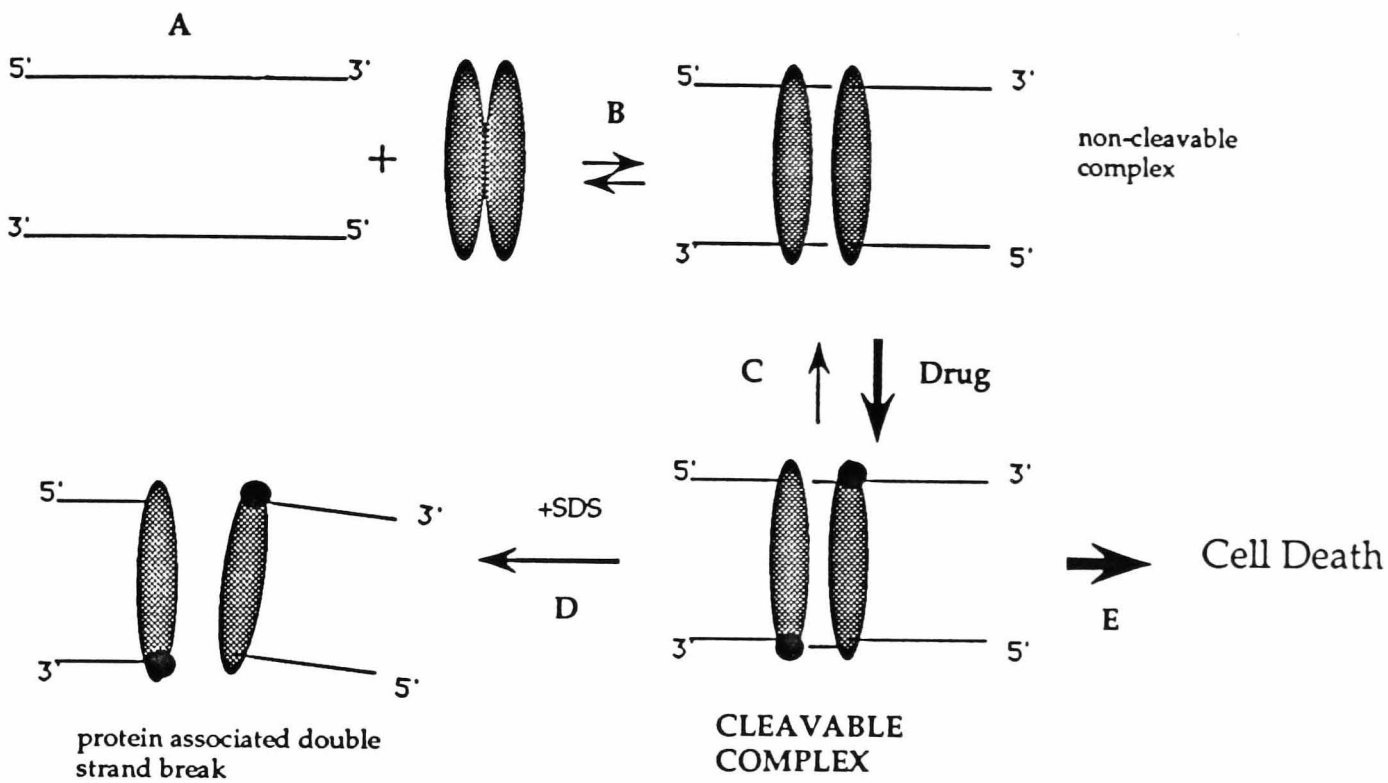


Figure 1.15. *A model for drug-induced topoisomerase II mediated DNA cleavage and cell death.*

A. Unbound topoisomerase II and substrate DNA.
B. Reversible formation of a non-cleavable topo II-DNA complex.
C. Reversible formation of a drug induced cleavable complex.
D. Revealing of a double strand break by SDS.
E. Cellular processing of cleavable complex resulting in cell death.

into a 'cell poison'.

1.7.3 *Type I topoisomerases*

These enzymes break one strand of the DNA helix, allowing the adjacent strand to pass through [or rotate around the phosphodiester bond opposite the nick] before resealing. The DNA linking number is changed by one. Topoisomerase I (reviewed by Gellert, 1981; Lock and Ross, 1987) is a single polypeptide with a molecular mass of 100,000 Da. It does not require ATP for its action (Liu, 1983). Topoisomerase I has also been shown to be a target for some antitumour agents, including camptothecin (Avemann *et al.*, 1988). The mechanism of action is analagous to that described for topoisomerase II targeting drugs, with formation of a cleavable complex [on a single strand] with the enzyme covalently bound to the 3' phosphoryl end of the break and a free 5' hydroxyl end [see figure 1.16]. Treatment with denaturants results in revealing of single strand breaks.

Cleavable complex formation results in formation of a bulky DNA-enzyme adduct, protein concealed strand breaks and an inhibition of enzyme function. It is not yet clear which factor is most important in expressing cytotoxicity. In addition, there is no clear understanding of how topoisomerase cleavable complexes are converted to lethal DNA strand breaks *in vivo*. Avemann *et al.* (1988) showed that camptothecin induced topoisomerase I mediated strand breakage at the replication forks of SV40 minichromosomes. Also, Schneider *et al.* (1989) found that mAMSA was maximally cytotoxic in cells undergoing RNA and protein synthesis. When RNA and protein synthesis were inhibited mAMSA produced no change in the level of cleavable complexes formed, suggesting an active event is required for conversion of cleavable complex formation to cell kill (Schneider *et al.*, 1989). Topoisomerases have also been implicated in the regulation of gene expression. For example topoisomerase II cleavage sites have been identified in the regulatory flanking sequences of a number of genes including heat shock genes (HSP70) (Yang *et al.*, 1985) and the enhancer sequence of immunoglobulin kappa and other genes

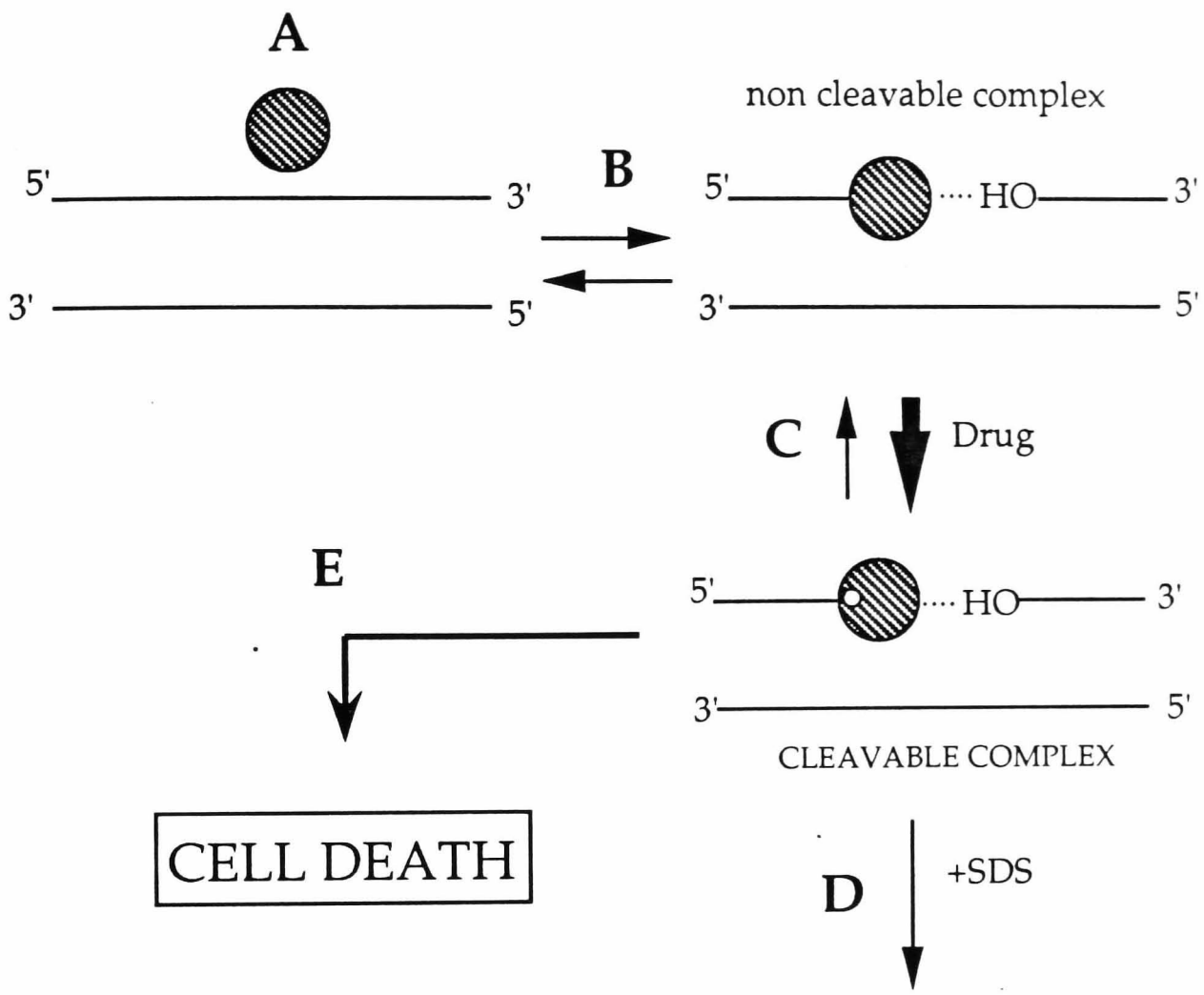


Figure 1.16. *A model for drug-induced, topoisomerase I mediated DNA cleavage and cell death*

A. Unbound topo I and substrate DNA
B. Reversible formation of a noncleavable topo I-DNA complex.
C. Reversible formation of a drug-induced cleavable complex.
D. Revealing of a single strand break by SDS
E. Cellular processing of cleavable complex leading to cell death

o = covalent linkage

(reviewed by Bodley and Liu, 1988). Topoisomerase II has also been implicated in the expression of c-fos oncogene (reviewed by Bodley and Liu, 1988). The topoisomerase inhibitor novobiocin has been shown to induce expression of heat shock genes in *Drosophila* (Francis *et al.*, 1987). Similarly, VM26 was found to induce heat shock proteins via interaction with topoisomerase II (Rowe *et al.*, 1986). The topoisomerase I inhibitor camptothecin was found to inhibit heat induced transcription of the HSP70 heat shock gene via interaction at the DNase hypersensitive sites of this gene (Rowe *et al.*, 1986). Furthermore, camptothecin mediated DNA strand breakage has been located near to transcriptionally active genes (reviewed by Bodley and Liu, 1988). mAMSA has been found to mediate DNA cleavage at the c-myc proto-oncogene locus (Riou *et al.*, 1986). Topoisomerase I (Stevsner *et al.*, 1989) and topoisomerase II (reviewed by Glisson and Ross, 1987) have been shown to bind to specific DNA sequences. Topoisomerase I (Lim *et al.*, 1986) and topoisomerase II inhibitors (Dillehay *et al.*, 1987; Anderson and Kihlman, 1989) have also been shown to result in an increase in DNA sister chromatid exchanges which result in a disruption of gene expression. Such sister chromatid exchanges could be responsible for the lethality of drug-induced cleavable complexes. This has been proposed to be due to exchange of topoisomerase subunits on adjacent DNA strands (Pommier *et al.*, 1985).

Thus, topoisomerases are likely to be involved in the expression and activation of oncogenes. Oncogenes have been shown to be involved in tumourigenesis and the maintenance of the transformed phenotype of tumour cells (reviewed by Bishop, 1987) [see section 1.2.2]. A link is therefore being established between antitumour drug interactions with topoisomerases and effects on the expression of oncogenes. Therefore, it is likely that the role of topoisomerases in the control of cell growth, cell division and the maintenance of the tumourigenic state is the key feature of this enzyme being a target for antitumour agents in proliferative tumour tissue as opposed to normal tissues.

As described in section 1.6.3 antitumour agents can bring about damage to DNA and other cellular macromolecules by the formation of reactive drug intermediates or by the formation of oxygen free radicals. In the

next section the cellular events which lead to these processes are described as well as their effects on macromolecular targets within the cell.

1.8 Free radical generation by antitumour agents

Before the formation of oxygen free radicals by antitumour drugs is ccnsidered the nature, chemistry and biological consequence of these radicals needs to be considered.

1.8.1 *The chemistry of oxygen free radicals*

A free radical is defined as " a chemical entity which contains one or more unpaired electrons and is capable of an independent existance". An unpaired electron is one that individually occupies one atomic or molecular orbital.

Ground state oxygen has two unpaired electrons of parallel spins [figure 1.17] each one located in a different Pi* antibonding orbital and is therefore a free radical. However, ground state oxygen is not very reactive as an oxidising agent since it can only accept electrons one at a time due to the spin restriction of the unpaired electrons as they both have parallel spin.

Singlet oxygen [figure 1.17], which is a more reactive form of oxygen which is formed by input of energy into ground state oxygen. $^1\Delta gO_2$ is not a radical, having an energy 22.4 Kcal above the ground state. $^1\Sigma g^+$ is even more reactive [37.5 Kcal above ground state], but usually decays rapidly to $^1\Delta gO_2$ before reacting with other chemical entities. $^1\Sigma g^+$ state can be produced by illumination of biological pigments such as flavins and porphyrins in the presence of oxygen [reviewed by Halliwell and Gutteridge, 1984].

If a single electron is added to ground state oxygen it must enter one of the Pi* antibonding orbitals producing the superoxide anion radical [O_2^-]. This has a short life span under aqueous conditions and undergoes dismutation by reaction (1). The first step in this reaction

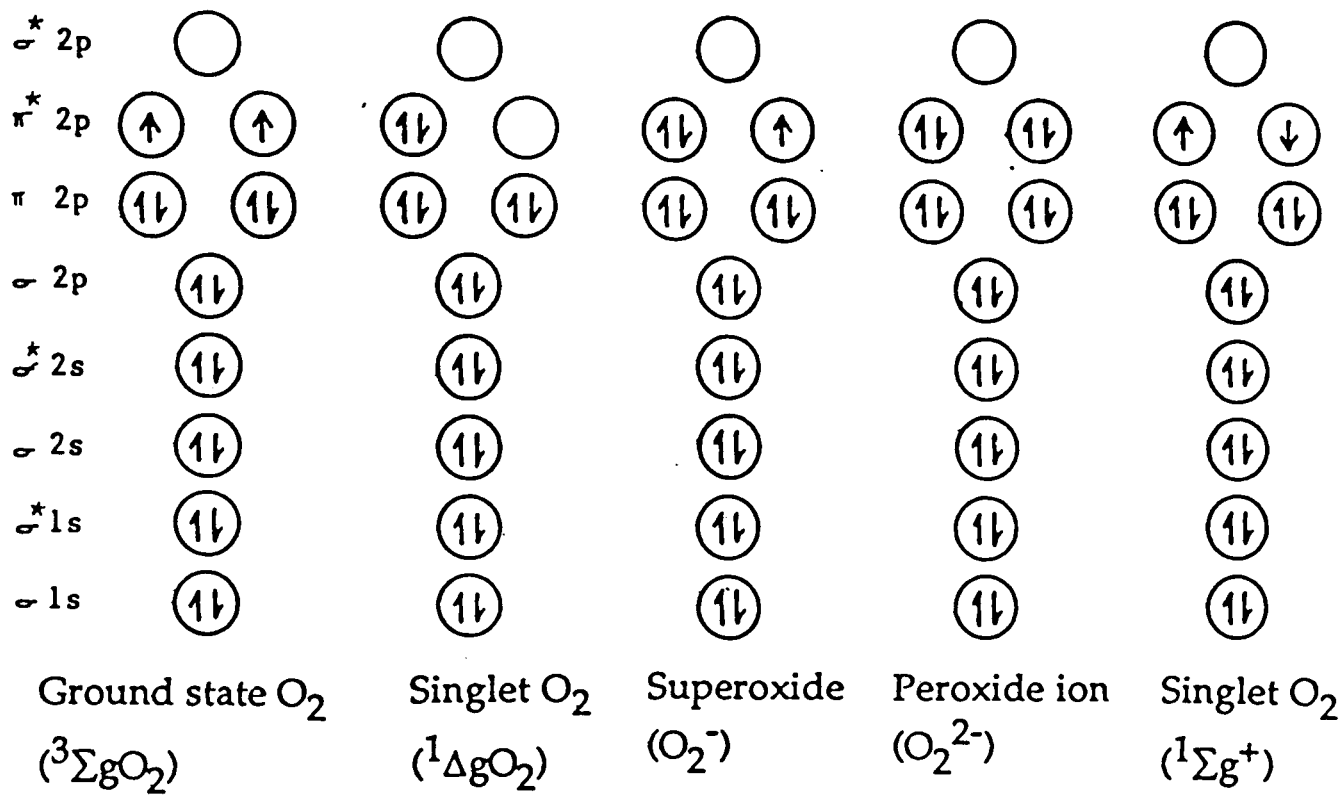


Figure 1.17 *Bonding in the diatomic oxygen molecule.*

is the protonation of superoxide anion to form a hydroperoxyl radical [$HO_2\cdot$].

$$HO_2\cdot + O_2^- \longrightarrow HO_2^- + O_2 \xrightarrow{+H_2O} O_2 + H_2O_2 + OH^- \qquad (1)$$

This reaction has a rate constant of 10^5 $M^{-1}S^{-1}$, which is increased to 10^9 $M^{-1}S^{-1}$ in the presence of superoxide dismutase (Koppenol and Butler, 1985).

Addition of a second electron to ground state oxygen gives the peroxide ion [O_2^{2-}] which is not a free radical. The occupation of antibonding orbitals by electrons in the superoxide anion and peroxide radical results in a weakening of the O-O bond. Addition of two further electrons to the peroxide ion (which enter σ^*2p orbitals) results in destruction of the O-O bond and formation of two O^{2-} species. In biological systems the peroxide ion is usually protonated to form hydrogen peroxide [H_2O_2](equation 2). Hydrogen peroxide is stable and a good oxidant. Addition of a further two electrons to the peroxide ion results in the formation of water (equation 3).

$$O_2 \xrightarrow{+2e^- \ +2H^+} H_2O_2 \qquad (2)$$

$$O_2 \xrightarrow{+4e^- \ +4H^+} 2H_2O \qquad (3)$$

The hydroxyl radical [$\cdot OH$] is formed by the homolytic fission of the O-O bond in hydrogen peroxide. This can be achieved by heat, ionising radiation or a mixture of H_2O_2 and transition metal salts especially iron (II) and copper salts [Fenton reaction (reaction 4)] (reviewed by Walling, 1975). The hydroxyl radical is a highly reactive and potent oxidant and has a very short life span.

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH\cdot + OH^- \qquad (4)$$

The generation of oxygen radicals can have serious implications in the cellular environment, as will be described in section 1.9. Formation of oxygen radicals is believed to be involved in the cytotoxic effect of a number of antitumour agents and hence has important therapeutic implications.

1.8.2 *Free radical formation by quinone antitumour agents*

A characteristic feature of the quinone moiety is the ability to undergo reversible oxidation-reduction. Simple antitumour quinones [eg 2-methyl-1,4-napthoquinone (menadione)] (figure 1.18) can undergo one electron reduction to a semiquinone free radical ($QH^{\cdot}$) [equation 5] or two electron reduction to a hydroquinone (QH_2). A semiquinone can also be formed by comproportionation between a quinone and a hydroquinone (equation 6).

$$Q \longrightarrow QH^{\cdot} \qquad (5)$$

$$QH_2 + Q \rightleftharpoons 2QH^{\cdot} \qquad (6)$$

These reductions can occur non-enzymatically under physiological conditions by reduction with pyridine nucleotides, with cellular thiols (eg glutathione), by electron transfer from less electron affinic semiquinones or from an oxygen free radical such as superoxide anion (reviewed by Powis, 1989).

Quinones can also undergo enzymatic reduction by flavin containing enzymes, resulting in formation of semiquinone free radicals (see figure 1.19). Enzymes which can catalyse one electron reduction of quinones include NADPH cytochrome P450 reductase and NADH cytochrome b_5 reductase which are the major enzymes involved in reduction of quinones. These enzymes normally catalyse transfer of electrons from reduced

nicotinamide adenine dinucleotide phosphate (NADPH) and reduced nicotinamide adenine dinucleotide [NADH] to cytochrome P450 and cytochrome b_5 respectively. These reductases form the flavoprotein component of the mixed function oxygenase system located in the endoplasmic reticulum of most cell types. The term cytochrome P450 encompasses a range [Johnson, 1979] of monooxygenases that metabolise a wide range of xenobiotics by aromatic and aliphatic hydroxylation, nitrogen, oxygen and sulphur dealkylation, sulfoxidation and epoxidation [Orrenius and Ernster, 1974]. The physiological function of the monooxygenases is the metabolism of steroids. The efficiency of electron transfer from the reductases to the cytochrome component is absolute so the reductases can reduce other electron acceptors such as quinones (Bachur *et al.*, 1979) and quinoneimines (Van De Straat *et al.*, 1987).

Other enzymes that can catalyse the simultaneous one and two electron reduction of quinones include Xanthine oxidase, a cytosolic enzyme which is involved in the degradation of purines (requiring the cofactor NADH), NADH-lipoamide oxidoreductase, a member of the pyruvate dehydrogenase complex which oxidises pyruvate to acetyl Co A [reviewed by Stryer, 1988] and NADH dehydrogenase, which is involved in electron transport in the mitochondrial electron chain (transfers electrons to ubiquinone) [reviewed by Nicholls, 1981].

NADPH oxidoreductase (formerly termed DT diaphorase) [Lind, 1982], catalyses only two electron reduction of quiones to hydroquinones. This enzyme is involved in vitamin K metabolism. The conversion of the quinone directly to the hydroquinone is thought to bypass toxic effects associated with semiquinone formation [section 1.9]. However the hydroquinone can form the semiquinone by reaction with the quinone by comproportionation [equation 6]. Under aerobic conditions the semi-quinone autoxidises to form the superoxide anion and regenerate the quinone. The reaction with O_2 is rapid with a rate constant [typically 4.8×10^6 $M^{-1}sec^{-1}$] which depends on the type of quinone. The quinone becomes available for further enzymic reduction in a futile cycle of events. This process of repeated semiquinone formation and autooxidation back to the quinone is termed redox cycling (reviewed by

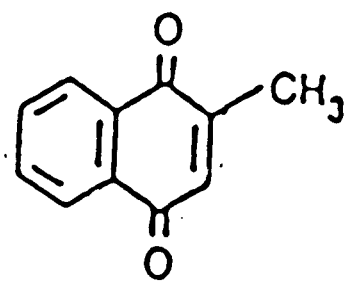
Hochstein, 1983, and Smith, 1985). The rate limiting step of superoxide anion formation is enzymic reduction of the quinone. This depends on the substrate specificity for the enzyme as well as its reduction potential. The above reactions are controlled by strict equilibria between formation of the semiquinone and re-formation of the quinone on reaction of the semiquinone with molecular oxygen (equation 7]. The direction of the reaction depends on the concentration of the semiquinone, molecular oxygen and the rate constants k_1 and K_{-1}.

$$Q + O_2^- \underset{K_{-1}}{\overset{K_1}{\rightleftharpoons}} Q^\cdot + O_2$$

Types of quinones that undergo enzymic reduction to semiquinones and redox cycling (reviewed by Powis, 1989) include menadione (Smith, 1985), anthraquinones (reviewed by Powis, 1989), anthracyclines (reviewed by Gianni, 1983), mitomycin C (Hoey *et al.*, 1988; reviewed by Pritsos and Sartorelli, 1986) and aziridinylbenzoquinones [eg AZQ (Gutierrez *et al.*, 1985 and 1986; Lea *et al.*, 1988) and BZQ (Butler *et al.*, 1989)], Fe(III)-bleomycin (Burger *et al.*, 1981; Scheulen *et al.*, 1981; Turner *et al.*, 1989), quinoimmines [eg Actinomycin D (Powis *et al.*, 1987; Flitter and Mason, 1988)] and Lapachols (reviewed by Powis, 1989). Figure 1.18 shows the structures of some of these agents.

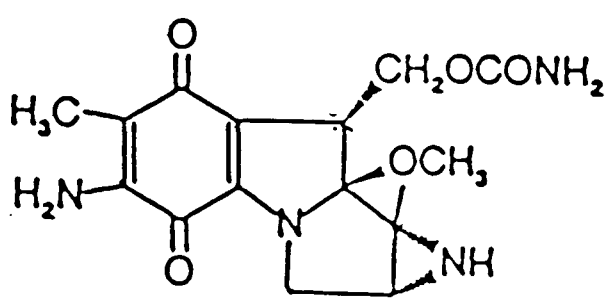
1.8.3 *Formation of superoxide anions a precursor for production of other oxygen radicals*

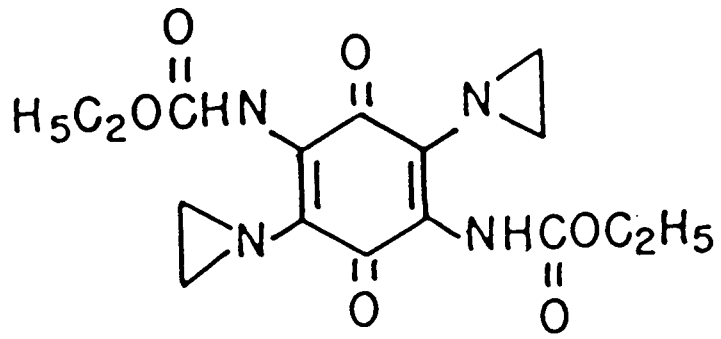
The formation of superoxide anions by quinone redox cycling can lead to a cascade of oxygen free radicals with potentially injurious or lethal consequences to the cell. The superoxide anion can act as an oxidant but is a better reductant (McCord, 1979). The dismutation of this unstable free radical either spontaneously [equation 1] or catalysed by superoxide dismutase, results in formation of hydrogen peroxide. This species is a fairly stable oxidant which can diffuse away from its site



Menadione

Mitomycin C





AZQ

Figure 1.18 *The structure of some redox active antitumour agents.*

of formation through cellular membranes. In the presence of trace amounts of iron (II) salts hydrogen peroxide reacts with superoxide anion to form the hydroxyl radical by the so-called iron catalysed Haber-Weiss reaction [equation 7, 8 and 9] (reviewed by Halliwell and Gutteridge, 1985).

$$Fe^{3+} + O_2^{-} \rightleftharpoons Fe^{2+} + O_2 \qquad (7)$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH\cdot + OH^{-} \qquad (8)$$

$$\text{net:}\quad O_2^{-} + H_2O_2 \longrightarrow O_2 + OH^{-} + HO\cdot \qquad (9)$$

Regeneration of iron (II) required for further iron catalysed Haber-Weiss reaction can be carried out by superoxide anion [equation 7] or a semiquinone free radical under anaerobic conditions [equation 10] (reviewed by Powis, 1989).

$$QH\cdot \xrightarrow{-H} Q + Fe^{2+} \qquad (10)$$

The Haber-Weiss reaction can also give rise to singlet oxygen [McCord, 1979] (equation 11).

$$O_2^{-} + H_2O_2 \xrightarrow{Fe} OH\cdot + OH^{-} + {}^1\Delta gO_2 \qquad (11)$$

These reactions can also be catalysed by copper salts [equations 12, 13, 14, 15 and 16], with resultant hydroxyl radical formation (reviewed by Sutton and Winterbourn, 1989).

$$Cu^{2+} + O_2^{-} \rightleftharpoons Cu^{+} + O_2 \qquad (12)$$

$$Cu^{+} + O_2^{-} \longrightarrow Cu^{2+} + O_2^{2-} \qquad (13)$$

$$O_2^{2-} + 2H^{+} \longrightarrow H_2O_2 \qquad (14)$$

$$\text{net: } O_2^{-} + O_2^{-} + 2H^{+} \longrightarrow H_2O_2 \qquad (15)$$

Additionally:-

$$Cu^{+} + H_2O_2 \longrightarrow Cu^{2+} + OH^{-} + OH^{\cdot} \qquad (16)$$

The hydroxyl radical is a highly reactive and potent oxidant which will react immediately with the nearest cellular component upon its formation, causing damage [see section 1.9]. A schematic diagram of quinone redox cycling and resultant reactive oxygen formation is shown in figure 1.19. The formation of oxygen free radicals by redox cycling of antitumour quinones is associated with a number of toxic consequences which individually or in combination may lead to cell death.

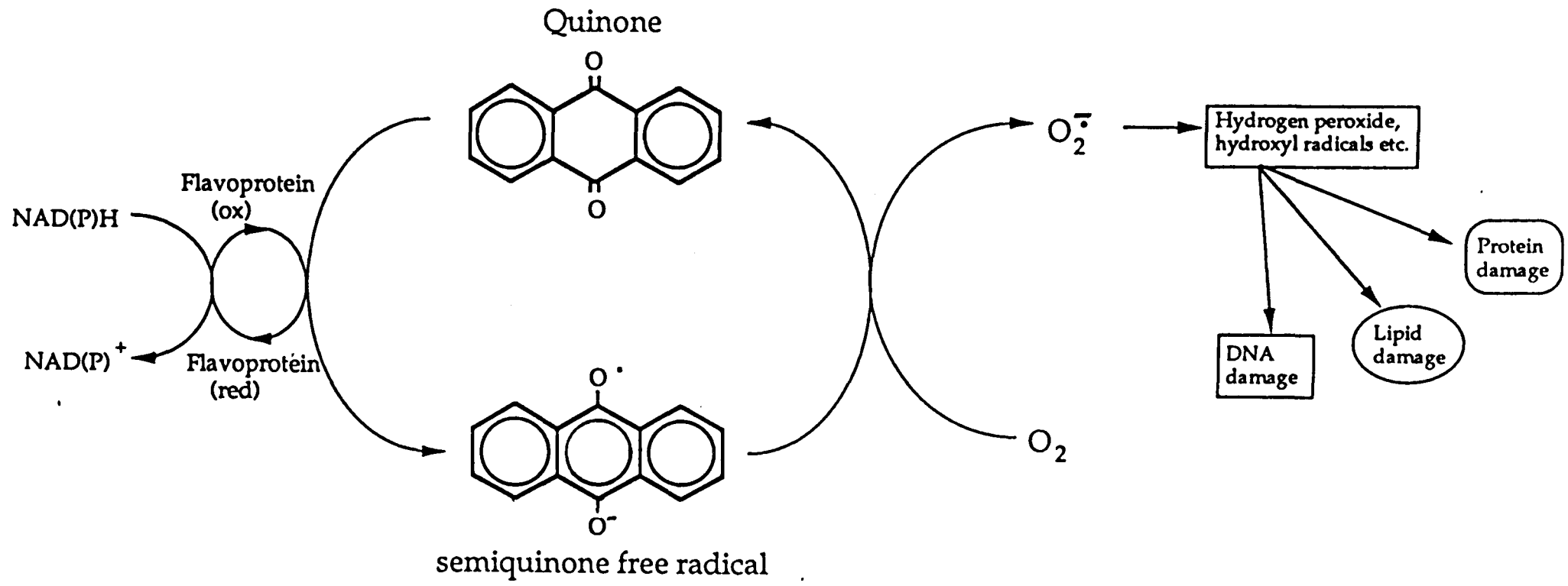


Figure 1.19 *Reductase mediated redox cycling of quinone antitumour agents.*

1.9 Consequences of oxygen free radical generation in cells

1.9.1 *Protein damage and inactivation*

Exposure of proteins to oxygen free radicals has been shown to result in aggregation and fragmentation (Davies, 1987), amino acid modification (Davies *et al.*, 1987a), modification of secondary and tertiary structure (Davies and Delsignore, 1987) and an increase in proteolytic susceptibility (Davies *et al.*, 1987b). Intracellularly, this could lead to inactivation of vital proteins such as enzymes. Oxygen free radicals have been shown to oxidise the sulphydryl groups of functional and structural proteins including enzymes (Slater, 1984). This process involves hydrogen abstraction from the thiol groups of the protein (Slater, 1984).

1.9.2 *Lipid peroxidation*

Lipid peroxidation (shown in figure 1.20) is defined as the oxidative deterioration of polyunsaturated lipids. This process involves the reaction of oxygen free radical species with polyunsaturated lipids to form lipid radicals and semi-stable lipid hydroperoxides (reviewed by Bus and Gibson, 1979; Halliwell and Gutteridge 1985). Lipid peroxidation can be divided into three distinct stages:-

a) Initiation:- Polyunsaturated lipids are susceptible to lipid peroxidation as the presence of a double bond weakens the C-H bond on the adjacent carbon atom to the unsaturated C=C bond. These allylic hydrogens are therefore considered as being partially activated and susceptible to abstraction by oxidants such as singlet oxygen and hydroxyl radical. The product of initiation is a lipid radical ($L^{\cdot}$)[equation 1].

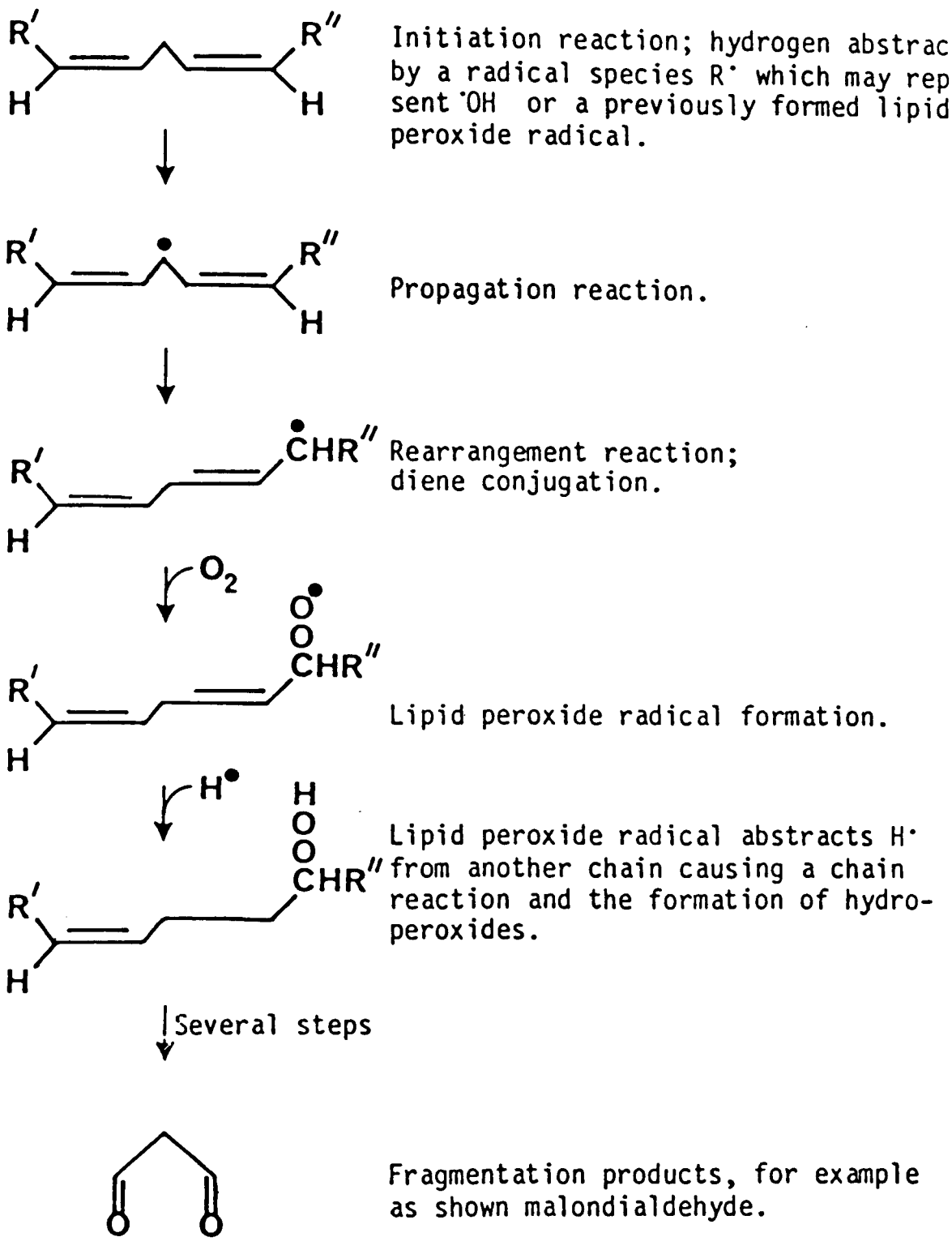


Figure 1.20 *The process of lipid peroxidation.*

$$LH \xrightarrow{-H} L^{\cdot} \qquad (1)$$

There is some evidence which shows that initiation is carried out by an iron chelate catalysed Haber-Weiss reaction as in equation 2 and 3 [reviewed by Halliwell and Gutteridge, 1984].

$$O_2^{-} + \text{ADP-Fe}^{3+} \rightleftharpoons O_2 + \text{ADP-Fe}^{3+} \qquad (2)$$

$$\text{ADP-Fe}^{2+} + H_2O_2 \longrightarrow \text{ADP-Fe}^{3+} + OH + OH^{-} \qquad (3)$$

In opposition to this, other workers propose that the initiating species is an ADP-perferryl ion complex [$\text{ADP-Fe}^{3+}\text{-}O_2$/ $\text{ADP-Fe}^{2+}\text{-}O_2^{-}$] (Minotti and Aust, 1987, Ursini *et al.*, 1989).

b) Propagation:- The lipid radical [L·] formed by initiation rearranges to form a conjugated diene [figure 1.20]. This then reacts with oxygen to give a lipid peroxy radical [LOO·] (equation 4). The peroxy free radical can abstract a hydrogen atom from another unsaturated lipid molecule forming a lipid hydroperoxide [LOOH] and another lipid free radical [L·] (equation 5). Thus another chain of lipid free radical formation is initiated.

$$L^{\cdot} + O_2 \longrightarrow LO_2^{\cdot} \qquad (4)$$

$$LOO^{\cdot} + LH \longrightarrow LOOH + L^{\cdot} \qquad (5)$$

Lipid hydroperoxides can decompose in the presence of transition metal complexes to form alkoxy and peroxy free radicals [equations 6 and 7 respectively].

$$LOOH + M^{n+} \longrightarrow LO^{\cdot} + OH^{-} + M^{(n+1)+} \quad (6)$$

$$LOOH + M^{(n+1)+} \longrightarrow LOO^{\cdot} + H^{+} + M^{n+} \quad (7)$$

c) Termination:- this involves formation of non free radical products [aldehydes, ketones, ethers, acids, ethane and pentane] by reaction of two lipid radicals, stopping the propagation process [equations 8, 9 and 10].

$$L^{\cdot} + L^{\cdot} \longrightarrow \text{non radical [stable] species} \quad (8)$$

$$L^{\cdot} + LOO^{\cdot} \longrightarrow \text{non radical [stable] species} \quad (9)$$

$$LOO^{\cdot} + LOO^{\cdot} \longrightarrow \text{non radical [stable] species} \quad (10)$$

1.9.2.1 *Consequences of lipid peroxidation*

The self propagating nature of lipid peroxidation can lead to oxidative stress in the cell with several possible consequences. However, it is difficult to dissociate causative lipid peroxidation and peroxidation due to cell death.

1. The intimate positioning of membrane lipids and proteins allows interaction between them. Lipid peroxidation can cause lipid-lipid and lipid-protein cross linking and in this way has been shown to inactivate enzymes and associated proteins including cytochrome P450 and calcium-ATPases [Kappus, 1985].

2. Peroxidation of membranes leads to cell and organelle permeability changes as a result of loss of structural integrity of the membrane (reviewed by Richter, 1987).

3. Lipid peroxidation products [radical and non radical] have been shown to cause damage to DNA (Vaca, 1988), including nucleic acid

base damage and ribose sugar ring opening and cleavage. The site of nuclear membrane-chromatin attachment is a possible site for lipid radical attack on DNA.

4. Production of lipid radicals places a demand on glutathione dependent detoxification mechanisms [section 1.10.1]. This system requires NADPH for reduction of oxidised glutathione. Thus cellular reducing equivalents can be depleted leading to a compromise of essential cellular processes and a reduced ability of the cell to cope with oxidative stress. In support of this glutathione depleting agents have been shown to increase lipid peroxidation in cells [Comporti, 1987]. Kappus, (1985) and Tribble *et al.* (1987) discuss the significance of quinone redox cycling with relation to lipid peroxidation.

1.9.3 *DNA damage caused by oxygen free radicals*

The formation of oxygen radicals has been shown to have many damaging effects to DNA structure and function and subsequent detrimental effects on cell survival. Most information on radical damage to DNA has been gained from studying the effect of X-ray and gamma radiation on isolated or cellular DNA. Quinone antitumour agents, hydrogen peroxide and other oxy-radical generating systems have been shown to produce similar effects to irradiation. In either case the critical species in DNA damage is considered to be the hydroxyl radical. With antitumour agents this is formed by redox processes via the iron catalysed Haber-Weiss reaction [see section 1.8.2]. Irradiation produces hydroxyl radicals via photochemical processes or by direct radiolysis of water molecules (reviewed by Ward, 1985).

The integrity of DNA is essential for the survival of cells. The cells of an organism are continually exposed to physical and chemical hazards that can lead to DNA damage. Consequently cells have a wide range of repair systems available to counteract this. In order to assess the consequences of DNA lesions, the availability and capacity of repair

processes needs to be considered.

1.9.3.1 *Formation of single [SSB] and double strand [DSB] breaks*

It has been proposed that hydroxyl radical formation can be 'site specific' being formed close to the DNA duplex by the iron catalysed Haber-Weiss reaction, catalysed by transition metal ions bound to the DNA (Ward, 1987; Chevion, 1988) [equation 1-5].

$$M^{+} + H_2O_2 \longrightarrow M^{2+} + OH^{\cdot} \qquad (1)$$

$$DNA + OH^{\cdot} \longrightarrow DNASSB \qquad (2)$$

$$M^{2+} \longrightarrow M^{+} \qquad (3)$$

$$M^{+} + H_2O_2 \longrightarrow M^{2+} + OH^{\cdot} \qquad (4)$$

$$DNASSB + OH^{\cdot} \longrightarrow DNADSB \qquad (5)$$

Equation 3 involves reduction of the metal ion by a free radical species such as superoxide anion or drug semiquinone or a cellular thiol such as glutathione (see section 1.10.1). A double strand break will be formed if after generation of a single strand break another hydroxyl radical is produced by the metal ion bound at this or an adjacent site resulting in 'multiple hits' on the DNA.

It is thought that hydroxyl radical attack on DNA occurs primarily by hydrogen abstraction from the 2' and 3' carbon atom of deoxyribose, followed by hydrolysis to generate an SSB [see figures 3.19 and 3.20]. Alternatively sugar modification can lead to purine/pyrimidine base loss and a resultant apurinic/ apyrimidinic site [see section 1.6.3.1 and figure 3.21]. Another site of reaction is addition to the carbon double

bond of DNA bases [see figure 3.22]. At the site of an SSB a deoxynucleotide is deleted leaving 3′ or 5′ monophosphate termini which are favourable for enzyme repair. However, saturation of the repair process may lead to lethal damage especially when the SSB is unstable and decays to an irrepairable state if not repaired quickly.

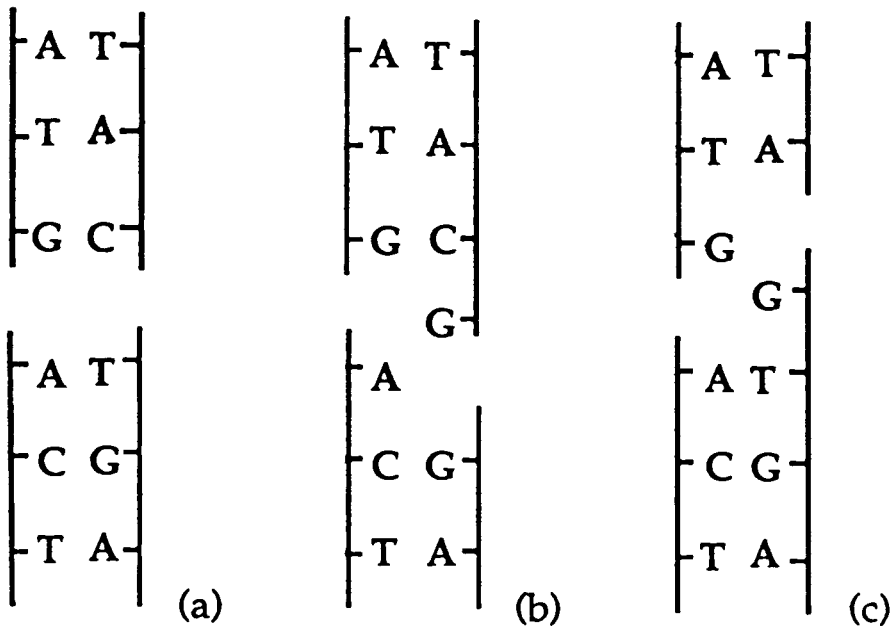
When two adjacent damaged sites occur on opposite strands the result is a DSB. This has a low probability compared to SSB. Alternatively, it is claimed that one hydroxyl radical can give rise to a DSB by radical transfer [interstrand H-transfer] from one strand to another [Schulte-Frohlinde, 1987].

Figure 1.21(a) illustrates a DSB which will possibly be lethal to the cell as repair processes cannot repair it effectively. Even if the strand break is resealed, base loss and hence a loss of DNA template integrity will occur. Additionally, the diffusion of the DNA strands away from each other may make repair difficult. Figure 1.21(b) and (c) illustrate staggered SSB. It is not known whether these breaks are repairable.

1.9.3.2 *Base damage or loss and strand breakage*

Reaction of DNA with the hydroxyl radical produces a series of base modifications (reviewed by Wallace, 1987). Pyrimidines, and in particular thymidine are more susceptible to hydroxyl radical attack than purine bases [see figure 3.22]. This is because hydroxyl radicals are electrophilic and hence when they react with carbon-carbon double bonds they react with the site of highest electron density. The C_5 position on pyrimidines is particularly vulnerable to hydroxyl radical addition with a C_6 carbonyl radical the result. The formation of base radicals has been confirmed by esr spectrometry (Cullis and Symons, 1986). Attack can also occur on the C_6 position resulting in a C_5 carbonyl radical. The hydroxyl radical adducts of the pyrimidine react rapidly with one another to form dimers, or disproportionation may

1. *Strand breakage*



2. *Base damage*

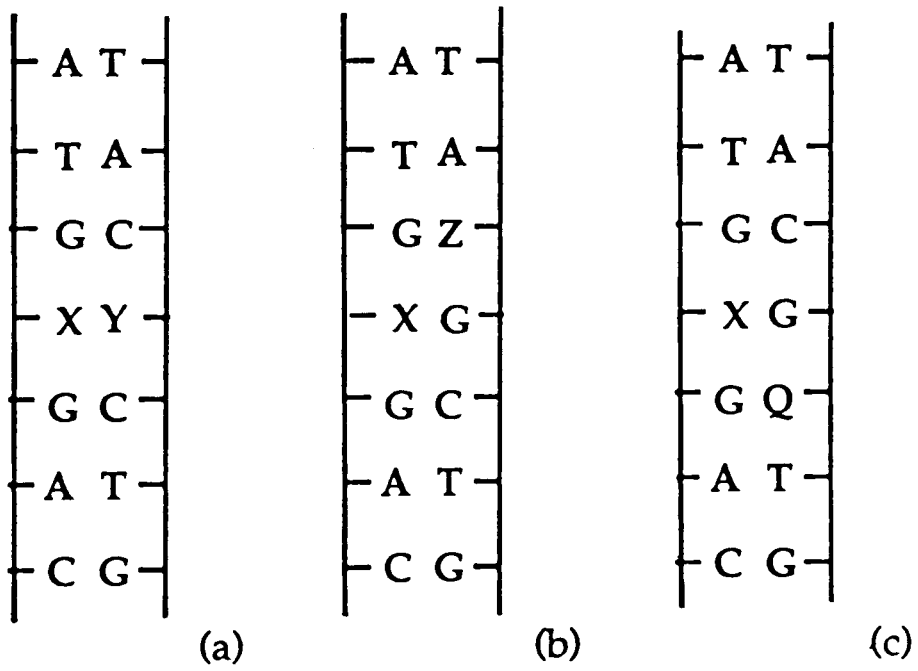


Figure 1.21. *Schematic representation of types of DNA strand breakage and base damage*

(Ward, 1985)

occur yielding a series of products (Dizdaroglu and Bergtold, 1986; Dizdaroglu *et al.*, 1987). The glycols predominate in these products. Molecular oxygen can react in a diffusion controlled manner with the carbonyl radicals forming peroxyl radicals, which on reaction with hydroxide ions at the N_1 position results in peroxyl anions. Elimination of superoxide anion from this yielding the corresponding isopyrimidine (Von Sonntag, 1988).

Purines also react at C_4, C_5 and C_8 with hydroxyl radicals giving rise to a number of unimolecular reactions such as ring opening and formation of radical cations [Von Sonntag, 1988; Teebor *et al.*, 1988]

The conversion of base radicals to modified bases can lead to decomposition or hydrolysis at the glycosidic linkage resulting in apurinic or apyrimidinic sites [AP's]. These sites are alkali labile and on treatment with alkali hydrolysis of the phosphodiester bond is promoted resulting in single or double strand breakage. These sites are susceptible to alkaline phosphatase endonucleases (AP) which can also result in strand breakage. The base radicals may also react with the sugar moiety to give strand breaks (Von Sonntag, 1988).

Altered DNA bases will be misread during DNA replication or transcription and are thus detrimental to the cell unless excised by specific endonucleases. In the case of figure 1.21(d) loss of information will be the result as bases on opposite strands are modified and will be excised. It is not known if offset base damage [figure 1.21 (e) and (f)] is repairable. In a similar manner to that described for strand breakage, one hydroxyl radical can damage complementary or neighbouring base pairs by radical transfer [Baverstock and Cundall, 1988].

1.9.3.3 *DNA interstrand, intrastrand and DNA-protein crosslinks*

Hydroxyl radicals can lead to DNA crosslinking or DNA-protein crosslinks [Teebor *et al.*, 1988]. Crosslinks between DNA and histones and other nuclear proteins have been identified (Oleinick *et al.*, 1987). This damage is also believed to occur due to formation of radical species within the DNA (or protein) molecule. The repair mechanism of such crosslinks has not been identified therefore crosslinking might represent lethal damage.

The net effect of the types of DNA damage described above is a disruption of DNA function which is likely to range from damage to a specific gene involved in essential cellular processes to disruption of DNA relication and RNA transcription. Section 1.6.3 describes DNA lesions caused by antitumour agents which are not initiated by radical processes.

1.9.3.4 *DNA repair mechanisms*

There are likely to be many types of enzyme involved in repair of oxidative damage to DNA. These include:- i) formamidopyrimidine-DNA glycosylase, which catalyses the release of potentially cytotoxic purine residues, ii) a DNA glycosylase which releases urea (a remnant of urea and several other derivatives with fragmented pyrimidine rings including thymine glycol (Doetsch *et al.*, 1986), iii) Mg^{2+} independent endonuclease which recognises apurinic sites on DNA (reviewed by Lindahl, 1987). A simplified scheme of DNA repair involves excision of the damaged base by a DNA glycosylase followed by removal of adjacent bases by a repair endonuclease which cuts the DNA phosphodiester backbone at the site of base excision. These events are followed by catalysis of DNA repair synthesis by DNA polymerase and resealing of the nick left in the repaired strand by a DNA ligase. These repair processes are also able to repair single, and with less efficiency, double DNA strand breakage (reviewed by Alberts, 1983; Bohr *et al.*,

1987). Other enzymes such as O^6-alkylguanine alkyl transferase can repair alkylated DNA sites produced by monofunctional alkylating agents and bifunctional alkylating agents (reviewed by Fox and Roberts, 1987).

1.10 Cellular protection against the formation of oxygen free radicals

1.10.1 *Enzymic defences*

Sections 1.8 and 1.9 describe the formation of oxygen free radicals and how they are detrimental to the cell. This subject area is reviewed by Ishikawa (1986) and Southern and Powis (1988). Two types of enzymes metabolise hydrogen peroxide in cells namely catalases and peroxidases.

1. Catalase is a a peroxisomal enzyme (reviewed by Halliwell and Gutteridge, 1985) and consists of four protein subunits, each of which contains a haem [Fe(III)-protoporphyrin] group bound to the active site. The reaction mechanism is:-

$$\text{Catalase-Fe(III)} + H_2O_2 \longrightarrow \text{Compound I}$$

$$\text{Compound I} + H_2O_2 \longrightarrow \text{Catalase-Fe(III)} + 2H_2O + O_2$$

Compound I is thought to be an Fe(IV) intermediate between Fe(III)-HOOH [ferric peroxide] and Fe(V)=O. This enzyme will oxidise a wide range of substrates including methanol and ethanol (reviewed by Mason and Chignell, 1982).

2. Glutathione peroxidase [reviewed by Meister and Anderson, 1983] catalyses the oxidation of reduced glutathione [GSH] to the oxidised form [GSSG] during the reduction of hydrogen peroxide and lipid peroxides to water and alcohols respectively (Niki, 1987):

$$H_2O_2 + 2GSH \longrightarrow GSSG + 2H_2O$$

$$2LOOH + 2GSH \longrightarrow GSSG + 2LOH$$

Phospholipase A is an enzyme which cleaves lipid peroxides from membranes which are then substrates for glutathione peroxidase. Glutathione peroxidase consists of four subunits each containing one atom of selenium at the active site in the form of selanocysteine which is the amino acid cysteine with the sulphur atom replaced by selenium. GSH reduces the selenium and the reduced form of the enzyme reacts with hydrogen peroxide. The ratios of GSH:GSSG need to be kept high by the cell in order to provide this protective mechanism. This is maintained by the glutathione reductases which consist of two protein subunits, each with the flavin FAD at the active site-

$$GSSG + NADPH + H^+ \longrightarrow 2GSH + NADP^+$$

NADPH reduces FAD which then reduces a disulphide bridge (S-S) located between two cysteine residues in the enzyme. The two protein-SH groups interact with GSSG and reduce it to two GSH groups. NADPH is provided by the pentose phosphate pathway which involves the action of the enzyme glucose-6-phosphate dehydrogenase (equation 1) followed by the action of 6-phosphogluconate (6-PG) dehydrogenase (equation 2).

$$\text{glucose-6-P} + NADP^+ \longrightarrow \text{6-phosphogluconate} + NADPH + H^+ \quad (1)$$

$$\text{6-PG} + NADP^+ \longrightarrow CO_2 + NADPH + H^+ + \text{ribulose-5-P} \quad (2)$$

As NADPH is oxidised, the pentose phosphate pathway or pentose phosphate 'shunt' increases its rate of production of NADPH. Besides acting in concert with glutathione peroxidase, glutathione can act directly as a scavenger of hydroxyl radicals and singlet oxygen. Additionally many toxic compounds are conjugated with GSH by glutathione-S-transferase enzymes during metabolism (reviewed by Moldeus and Quangan, 1987; Ross, 1988). Figure 1.22 illustrates the complex relationship of the glutathione pathway to other cellular

processes.

Superoxide dismutase [SOD] (reviewed by McCord, 1979 and Bannister *et al.*, 1987) was first identified by McCord and Fridovich (1969). Copper-zinc SOD is located in the cytoplasm and has a molecular weight of 32000 and contains two protein subunits each of which has an active site containing one copper ion and one zinc ion. Its function is to catalyse the dismutation of superoxide anions:-

$$O_2^- + O_2^- \xrightarrow{+2H^+} H_2O_2 + O_2 \text{ [ground state]}$$

The zinc ions [Zn^{2+}] do not participate in the catalysis but stabilises the enzyme. The copper ions function in this dismutation by undergoing alternate oxidation and reduction:-

$$\text{Enzyme-}Cu^{2+} + O_2^- \longrightarrow \text{Enzyme-}Cu^{+} + O_2$$

$$\text{Enzyme-}Cu^{+} + O_2^- + 2H^+ \longrightarrow \text{Enzyme-}Cu^{2+} + H_2O_2$$

$$\text{net: } O_2^- + O_2^- \longrightarrow H_2O_2 + O_2$$

Another type of SOD is also found in the mitochondrial matrix. This has four subunits each with 0.5-1.0 manganese ions per active site.

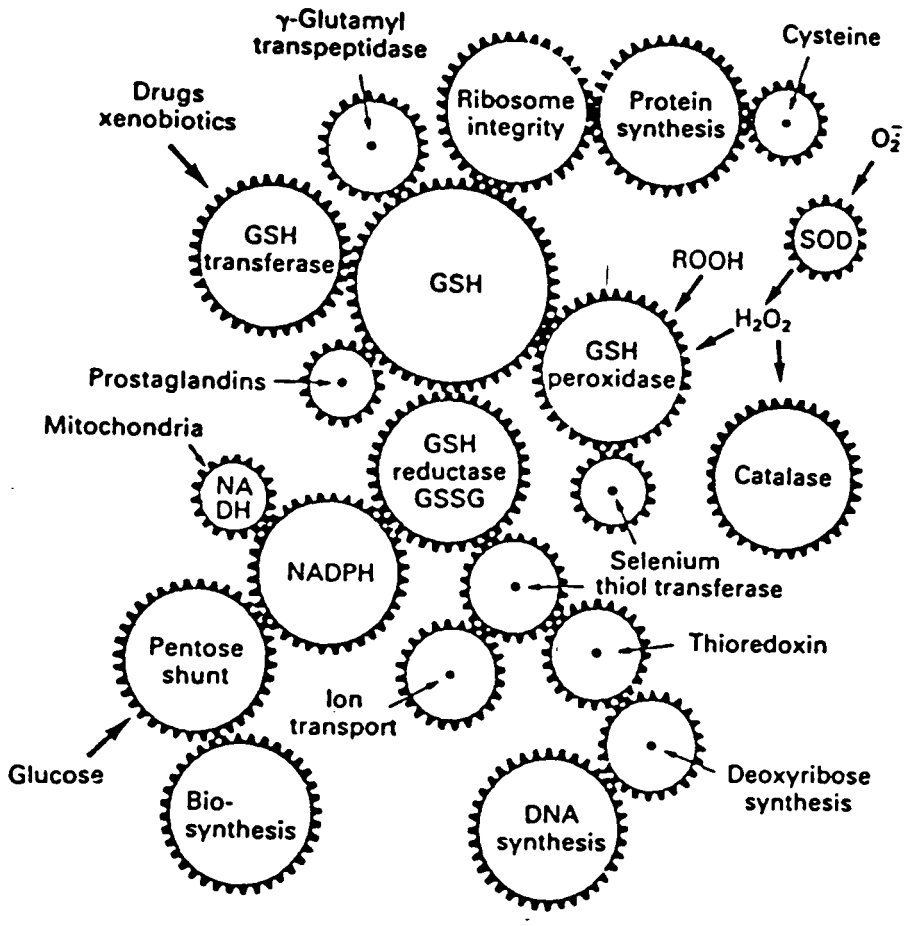


Figure 1.22 *Diagrammatic representation of the inter-relationship of glutathione [GSH] with other cellular systems.*

(Mitchell and Russo, 1987)

1.10.2 *Non-enzymic defences*

These are reviewed by Halliwell and Gutteridge (1984 and 1988). There are several important non enzymic antioxidants located in the cell. Ascorbic acid [vitamin C] is a cofactor for the enzymes proline hydroxylase, lysine hydroxylase and dopamine-beta-hydroxylase. However, it also acts alone as a reducing agent and can detoxify various organic radicals. Donation of one electron by ascorbate [figure 1.23] gives the semihydroascorbate radical. This can be further oxidised to dehydroascorbate. The semihydroascorbate radical is not very reactive and disproportionates:-

$$2 \text{ semihydroascorbate} \rightleftharpoons \text{ascorbate} + \text{dehydroascorbate}$$

Ascorbate will react rapidly with superoxide anion, hydrogen peroxide and hydroxyl radicals:-

$$\text{Ascorbate} + O_2^- + H^+ \longrightarrow H_2O_2 + \text{semihydroascorbate}$$

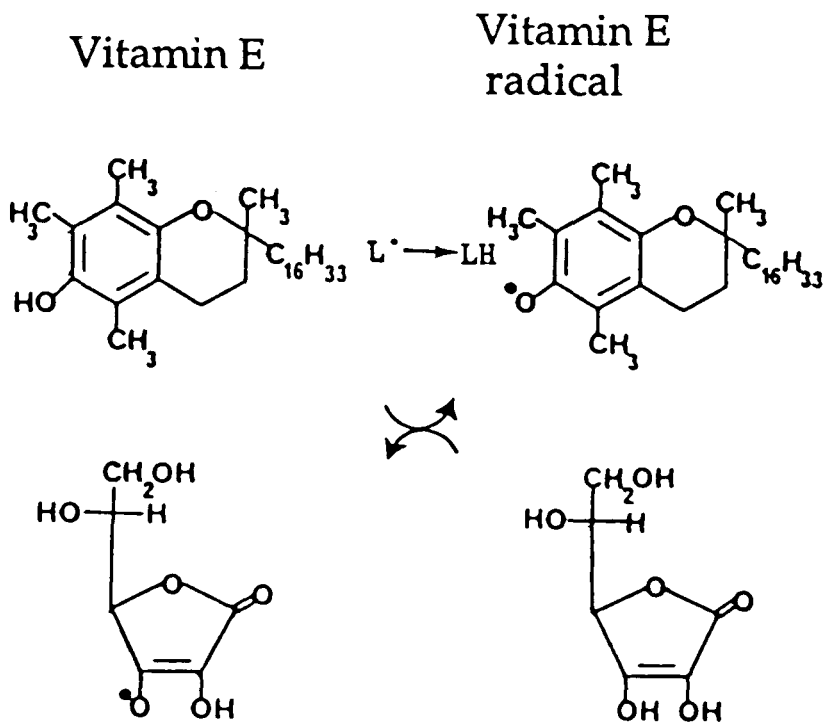
The enzyme dehydroascorbate reductase converts oxidised ascorbate back to the reduced form at the expense of GSH:-

$$2 \text{ dehydroascorbate} + 2GSH \longrightarrow GSSG + \text{ascorbate}$$

Alpha-tocopherol [vitamin E] is a lipid soluble antioxidant [figure 1.23] which protects against lipid peroxidation. Vitamin E reacts with lipid peroxy radicals to produce vitamin E radicals that are insufficiently reactive to participate in propagation reactions [equation 1]. Therefore vitamin E is termed a chain terminating antioxidant. The vitamin E radicals formed can be reduced back to vitamin E by ascorbate [see figure 1.23].

$$\text{Vitamin E} + RO_2\cdot \longrightarrow RO_2H + \text{Vitamin E}\cdot \qquad (1)$$

Other vitamin E type antioxidants include the sulphydryl group containing cysteine and cysteamine.



[L· = lipid radical]

Figure 1.23. *Interaction of Vitamin E and Vitamin C.*

1.11 Measurement of DNA damage in mammalian cells

Several assay methods are available for measuring DNA damage by antitumour agents *in vivo*. These include filter elution, HPLC determination of base damage for example detection of 8-hydroxyguanine (Floyd *et al.*, 1988; Kasai *et al.*, 1986), the fluorescent enhancement assay for DNA unwinding (Kanter and Schwartz, 1982), measurement of the viscosity of alkaline cell lysates (Marshall and Ralph, 1982)] and sedimentation of DNA fragments through alkaline sucrose gradients (Lett, 1981). Some of these techniques are useful in making a rapid determination of total strand breakage, or are useful in determination of specific types of DNA damage. The filter elution technique enables quantification of a wide range of different types of damage to DNA which is not possible by other techniques. Prior to the advent of this technique the most common method for single strand break determination was sedimentation of DNA through alkaline sucrose gradients using centrifugation. This technique however had various difficulties attached to its use (Lett, 1981). In recent years therefore the filter elution technique has come to the fore as a standard method for determining the extent and type of DNA damage by irradiation and other agents.

1.11.1 *Filter elution of DNA*

This technique, developed by Kohn *et al.* (1976) utilises filters to discriminate DNA strand breaks in mammalian cells produced by cytotoxic drugs or radiation. The filters are used as a mechanical barrier to the passage of large DNA strands. Variations in this technique allow determination of single strand breaks [SSB], DNA-protein cross links [DPC], DNA interstrand cross links [DIC], DNA double strand breaks [DSB] and protein concealed single strand breaks [PSSB] (Kohn *et al.*, 1976). The methodology is fully described in section 3.2.4. The elution rate of DNA (through the filter) isolated from cells treated with a particular agent is determined by collecting eluted samples of the DNA over a long time period and measuring the DNA content by

scintillation [if the DNA has been initially radiolabelled] (Kohn *et al.*, 1976) or fluorometrically using the DNA intercalating fluorescent dyes 3,5-diaminobenzoic acid dihydrochloride [DABA] (Kissane and Robins, 1958) or bisbenzamide [Hoechst 33258] (Erickson *et al.*, 1980).

1.12 Detection of Free radicals

1.12.1 *Biochemical methods*

There are two biochemical methods available for detection of superoxide anions in biological systems. The first method is dependent upon the reduction of acetylated cytochrome c by the superoxide anion, this reduction being followed by measuring the absorbance change at 550nm. Acetylation of more than 60% of the lysine residues of horse heart ferricytochrome c results in more than a 95% decrease in its ability to be reduced by microsomal reductases. However, its ability to be reduced by superoxide anion is maintained. Therefore this derivative is useful in detecting superoxide anions in systems containing cytochrome c reductases or oxidases. This assay is more specific if superoxide dismutase is used to confirm that acetylated cytochrome c is being reduced by superoxide anions (Azzi *et al.*, 1975).

The second method, the adrenochrome assay, is based on the oxidation of adrenaline to adrenochrome by superoxide anions (Misra and Fridovich, 1972) [see figure 1.24]. However, this assay has several disadvantages including the oxidation of adrenaline by hydroxyl radicals and autooxidation of adrenaline to adrenochrome (Heikkila and Cohen, 1973). This assay can be made more specific by the use of superoxide dismutase to confirm the dependence of the reaction on superoxide anions.

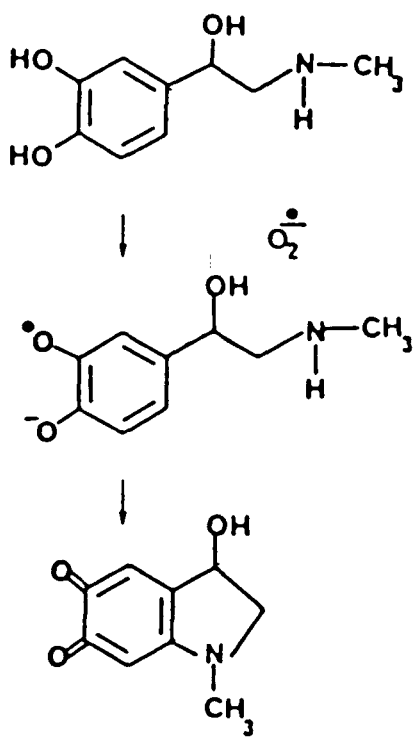


Figure 1.24. *Detection of superoxide anions by adrenochrome formation.*

1.12.2 *Electron spin resonance spectrometry*

1.12.2.1 *Principles*

Esr spectrometry (reviewed by Carrington and McLachlan, 1979; Foster, 1984) is based on the fact that the spin of an unpaired free electron produces a small but detectable magnetic moment. When an unpaired electron is subjected to an external magnetic field, the magnetic moment of the electron can take up two possible orientations, parallel or antiparallel with respect to the applied magnetic field and hence can occupy two different energy levels. Since electrons in the parallel orientation have lower energy, this is the preferred orientation. Electromagnetic radiation in the microwave frequency range can induce transitions or changes in spin orientation (resonance) from the lower to the higher energy level. This energy is equivalent to the energy difference between the two states [see figure 1.25]. Mathematically, this is expressed as the energy difference (ΔE) between the two spin states, which is proportional to the applied field, H, the value for the electron's magnetic moment, B and the spectroscopic splitting constant, g. Therefore, the equation for transition between energy levels for an unpaired electron is:-

$$\Delta E = g\beta H$$

An esr spectrometer detects the resonance of an unpaired electron by varying the magnetic field in relation to the microwave frequency. The first derivative of the absorption curve is recorded, representing a rate of change of absorption. This allows better sensitivity and resolution [see figure 1.26]. The area under the absorption curve is proportional to the effective spin population and is therefore indicative of the free radical concentration.

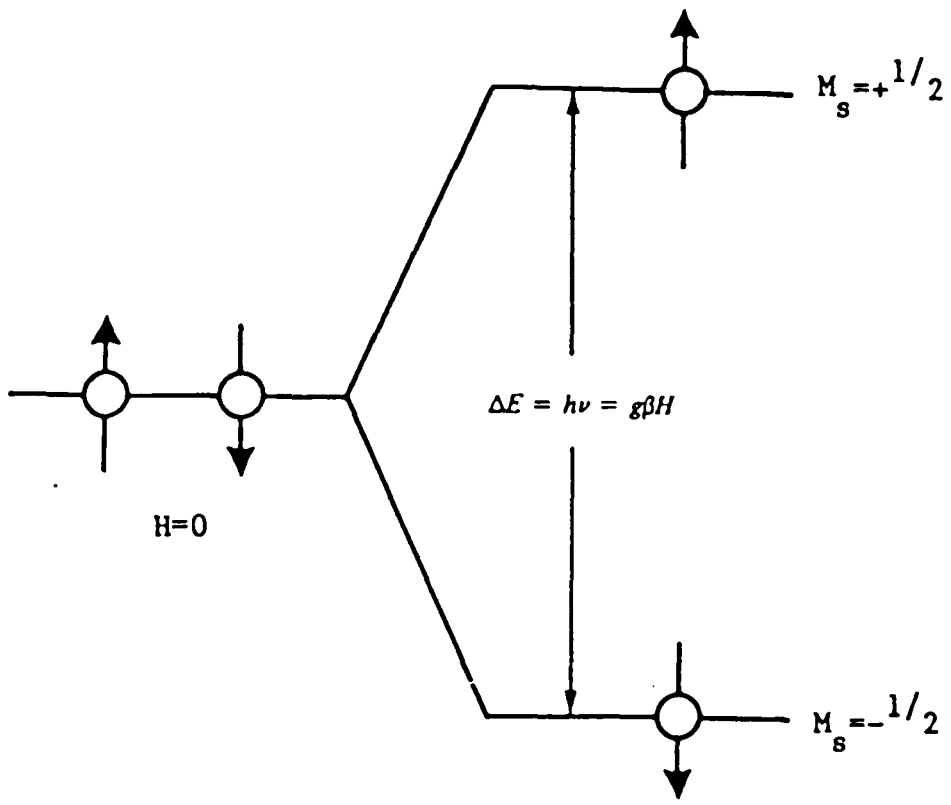


Figure 1.25. *The splitting of electron energy levels by a magnetic field*

The difference in the energy between the levels with opposed electron spin is $\Delta E = h\nu = g\,ßH$, where v is the frequency of the absorbed radiation, g is the spectroscopic splitting factor, ß is the Bohr magneton and H is a magnetic field. Ms are the spin states of the unpaired electron.

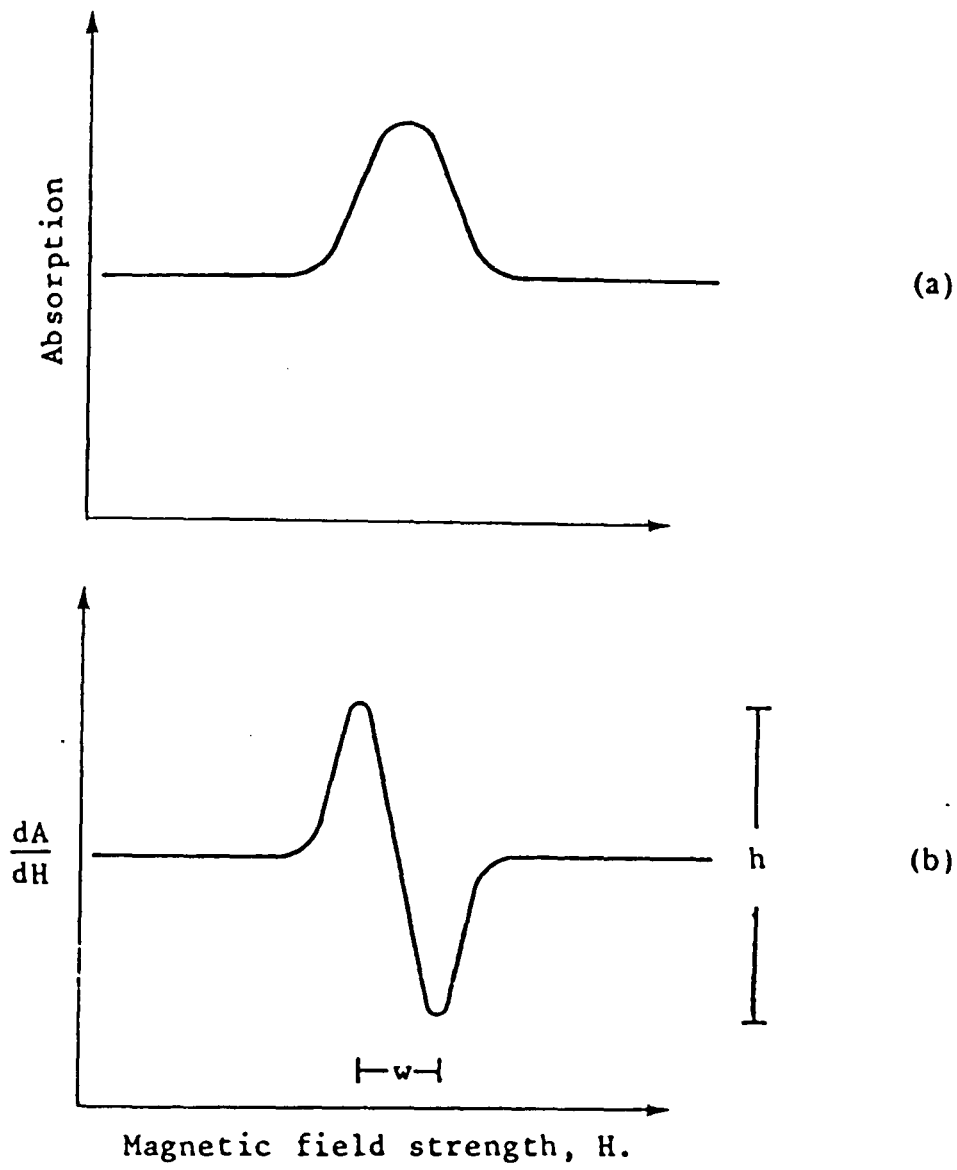


Figure 1.26. *Curves used in the presentation of electron spin resonance spectra.*

(a) the variation in the absorption of microwave power of the sample as the magnetic field is varied. (b) the first derivative of the microwave power absorption is plotted against the magnetic field to give the representation usually depicted for esr spectra. w and h represent the peak to peak width and height respectively. The area under the curve or peak to peak height for symmetrical first derivative curves is proportional to the concentration of free radicals.

1.12.2.2 *g-Value*

In a magnetic field an unpaired electron in a radical possesses, in addition to its spin angular momentum, a small amount of extra orbital angular momentum. The interaction between these, called spin-orbital coupling, results in the electron having a slightly different magnetic moment from that of the free electron . Consequently, the condition for resonance is altered, g being dependent on the radical. Hence, for a given frequency, radicals with different g values resonate at different field strengths, the g value being characteristic for a given radical.

1.12.2.3 *Detection of oxygen free radicals using spin trapping agents*

Free radicals can only be detected by esr if they are sufficiently long lived. However, oxygen free radical species are so reactive that they never reach the steady state levels required for detection by esr. Spin trapping techniques (reviewed by Mason, 1984; Samuni *et al.*, 1986; Pou *et al.*, 1989) can be used to overcome this problem. This method involves the use of a compound, usually a nitrone or nitroso compound [eg DMPO, figure 2.26] that can form a more stable nitroxide radical by reacting with short lived species such as superoxide anions and hydroxyl radicals. In this way the short lived species is 'trapped' as a species which can be detected directly by esr. In addition, hyperfine splitting constants of the adduct can provide information on the identity of the original radical [for example see figure 2.3]. Hyperfine splitting arises from the interaction between a nuclear magnetic moment with that of the unpaired electron [see figure 1.27]. The type of spin trap used depends on the lipophilic or hydrophilic nature of the environment in which the radical is generated.

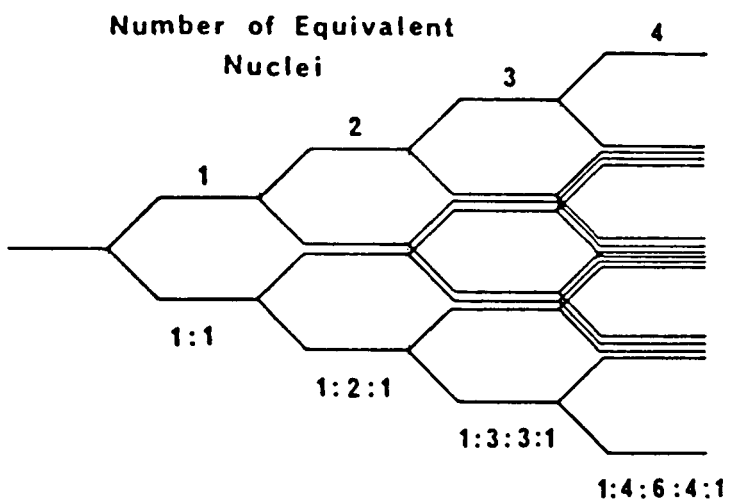


Figure 1.27. *The effect of interaction of the electron with increasing numbers of nuclear spin of 1/2. The bottom line shows the signal which would be observed, the intensity of each line of the signal being given by the figures beneath the splitting diagram.*

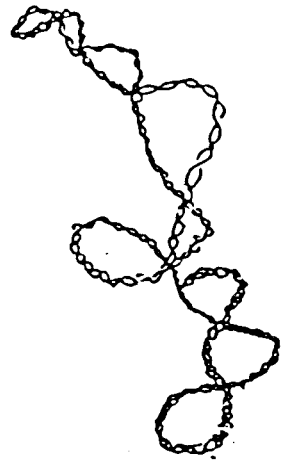
1.12.3 *Strand breakage of plasmid DNA as an indicator of free radical formation*

This assay utilises strand breakage of pBR322 plasmid DNA as an indicator of oxygen free radical formation by radiation, enzymically or chemically reduced quinone antitumour agents and other radical generating systems.

The genetic material of a bacterial cell is contained in a single double helical DNA molecule in the form of a closed circle. This 'chromosome' carries all the genes required for reproduction and propagation of the bacterium. Many bacteria also contain extra-chromosomal DNA in the form of small circles, known as plasmids. These carry genetic information for a variety of special functions including antibiotic resistance, toxin production and promotion of bacterial conjugation (reviewed by Stanier *et al.*, 1983). Plasmids can replicate independently of the chromosomal DNA, hence there may be many copies of a plasmid per bacterial cell. Plasmid pBR322 is found in Escherichia coli where it confers resistance to the antibiotics ampicillin and tetracycline. Plasmid pBR322 recA- is used for strand breakage studies as the presence of this repair enzyme causes formation of undesirable DNA species of different molecular weight [concatomers]. The basis for the isolation of this plasmid is described by Boffey (1983). The bacterial cells containing pBR322 are selected for by growing them in the presence of ampicillin. The amount of plasmid DNA can be enriched in relation to chromosomal DNA by selectively inhibiting chromosomal DNA replication. The plasmid DNA is separated from the chromosomal DNA by taking advantage of the large difference in molecular weight of the two species using isopycnic ultracentrifugation with a caesium chloride density gradient (reviewed by Williams and Wilson, 1983).

Plasmid pBR322 DNA in its native form consists of double stranded supercoiled [form I] DNA (figure 1.28). The supercoiling of this molecule places it under torsion. If one of the strands is nicked the strands unwind to give relaxed open circular [form II] DNA. If the

Form I (supercoiled) DNA



Form II (open circular or nicked) DNA

Form III (linear) DNA

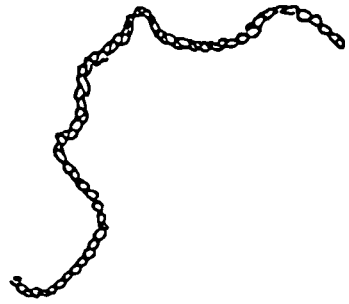


Figure 1.28 *The forms of pBR322 plasmid DNA.*

complementary strands are both nicked in an adjacent position [double strand nick] the result is linear [form III] DNA. These DNA species are resolved by submarine gel electrophoresis.

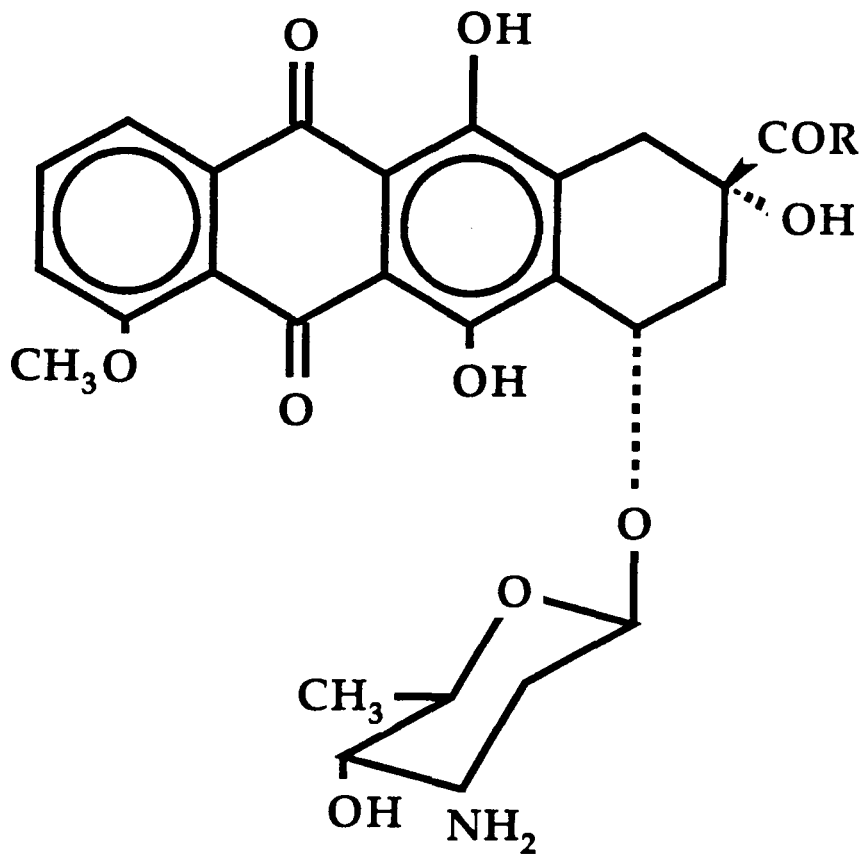
1.12.3.1 *Principles of agarose gel electrophoresis*

Agarose gels consist of a complex network of polymeric molecules and are used for separation of DNA fragments ranging from several hundred to 20,000 base pairs. At neutral or alkaline pH the DNA phosphate groups give rise to a uniform negative charge per unit length of the DNA molecule. Therefore in an electric field the DNA moves toward the positive pole, at a rate dependent on its size. If the molecules are in a gel they have to move through the pores of the gel as they move toward the positive pole, their speed depending on their size and shape. Thus the three forms of pBR322 DNA travel at different speeds through agarose gel. The highly compact form I DNA is the most mobile and travels fastest through the gel. This is followed by the rod shaped form III DNA which can travel 'end on' through the pores of the gel. The least mobile species is the non-rigid open circular form II DNA.

The separated DNA species in the gel are visualised by staining with the intercalating dye ethidium bromide (figure 1.11), as little as 0.05ug of DNA in one band can be detected as visible fluorescence upon illumination of the gel with ultra violet light (Old and Primrose, 1985). In order to quantify the DNA species on the gel a photograph is taken and the negative image analysed by scanning laser densitometry.

1.13 Development of the anthracyclines

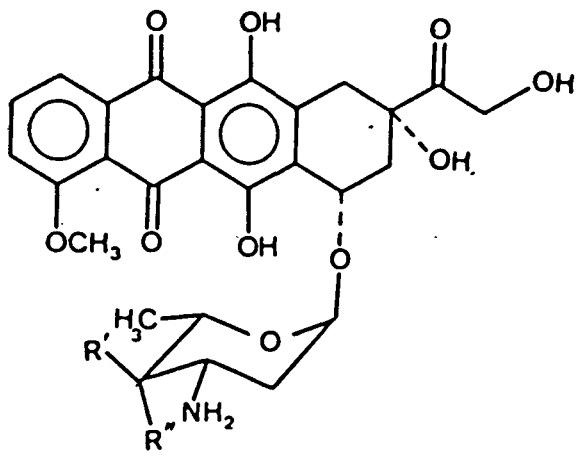
In the 1950's Farmitalia research laboratories, Milan initiated a programme to study anticancer compounds produced by bacteria isolated from soil. By 1957 a colony of Streptomyces caeroleorubidus which produced a red pigment was isolated. The antibiotic [daunorubicin] isolated from this strain was found to have potent antileukaemic activity. This agent was simultaneously discovered by Rhone Poulenc SA in France. Later the antitumour antibiotic Adriamycin [doxorubicin] was isolated from Streptomyces peucetius and found to have more potent antitumour activity and a wider spectrum of activity than daunorubicin. Both of these anthracycline antibiotics are characterised by a planar anthraquinone chromophore attached to an amino sugar- daunosamine [see figure 1.29]. By 1974, doxorubicin was on the market and has since been one of the most used antitumour agent in a wide range of human neoplasms [reviewed by Goodman, 1986] including leukaemia, lymphoma, multiple myeloma as well as solid tumours including breast tumours. However the use of doxorubicin has been associated with a cumulative dose limiting cardiotoxicity as well as myelosuppression. In consequence anthracyclines based on doxorubicin have been synthesised to investigate whether modifying the structural and stereochemical properties alleviate the toxic effects of and improve the therapeutic activity of this agent which is inactive in the treatment of melanoma, colon and renal carcinoma. New generation anthracyclines, which have promising antitumour activity but reduced cardiotoxicity, are currently undergoing clinical trials. These include anthracyclines modified at the aglycone moiety such as idarubicin, esorubicin, menogaril and epirubicin [see figure 1.30]. Epirubicin is the most promising of these analogues due to similar potency but reduced cardiotoxicity compared to doxorubicin (Johnston and Glazer, 1984)). Other anthracyclines, modified at the sugar moiety have been developed. Of these 4'-demethoxy-4'-O-methylodoxorubicin has proven to have a very high antitumour activity [see figure 1.31] (Arcamone, 1984).



R= CH_2OH doxorubicin

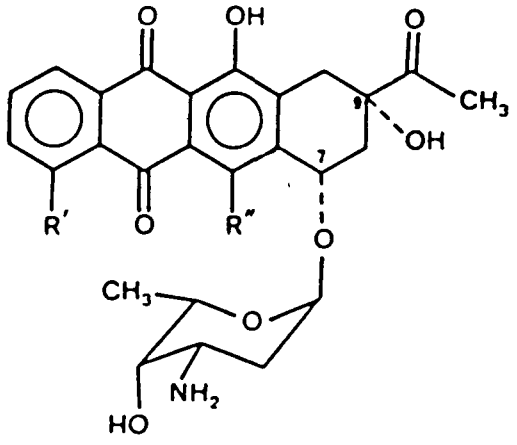
R= CH_3 daunorubicin

Figure 1.29. Structure of doxorubicin and daunorubicin



A : R'= OH, R"= H

B : R'= R"= H



C : R'=H, R"= OH

Figure 1.30 *Structures of epirubicin (A), esorubicin (B) and idarubicin (C).*

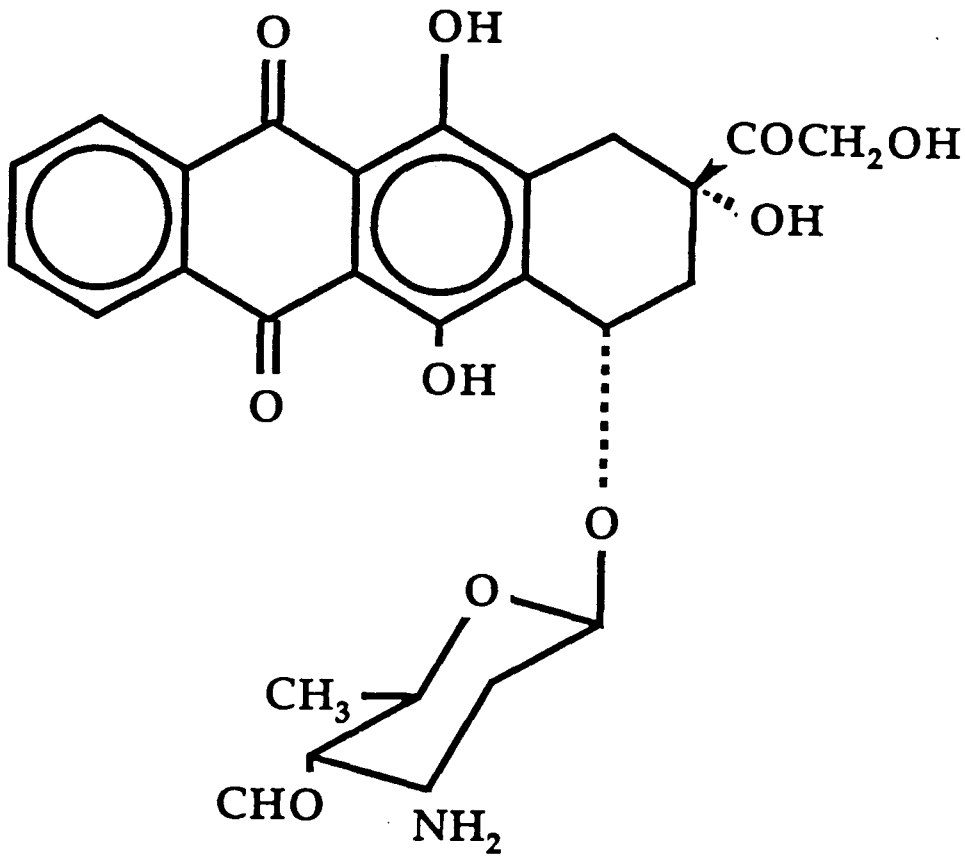


Figure 1.31 *Structure of 4-demethoxy-4-O-methylodoxorubicin*

1.14 Development of the anthraquinone antitumour agents

1,4-dihydroxy-5,8-bis((-[(2-hydroxyethyl)amino]ethyl)amino))-9,10-anthracenedione dihydrochloride [mitozantrone] (figure 1.32) is an anthraquinone antitumour agent which was discovered simultaneously by researchers at American Cyanid Company (Murdock *et al.*, 1979) and American Midwest Research Institute (Johnson *et al.*, 1979). Mitozantrone originated from a molecule designed to have structural features predicted to favour DNA intercalation (planar tricyclic chromophore). Mitozantrone was the best compound out of hundreds of anthraquinones synthesised. Mitozantrone maintains the N-O-O triangular pharmacophore which exists between the oxygens of the aglycone and the nitrogen of the amino sugar found in drugs such as doxorubicin. This feature was found to be required for antitumour activity (Zee Cheng and Cheng, 1983). However, the amino sugar of doxorubicin was considered to be associated with side effects such as cardiotoxicity [Traganos, 1983). The removal of the aminosugar and the use of basic side chains in mitozantrone was intended to diminish cardiotoxicity without altering the N-O-O triangle. The structural modification in mitozantrone which was found to have the greatest effect on antitumour activity was the presence of the aromatic hydroxyl groups on the 5 and 8 positions of the aromatic ring and the presence of the hydroxyethyl groups on the basic side chains.

Mitozantrone was found to be cytotoxic both to proliferative and non-proliferative tumour cells and had reduced cardiotoxic effects in animal systems (Smith, 1983). Clinical studies began in 1979 and since then mitozantrone has found to be useful in the treatment of breast carcinoma, acute leukaemia and lymphoma. However, side effects include myelosuppression and mucositis, although it has reduced gastrointestinal and cardiotoxic effects compared to doxorubicin (Lenk *et al.*, 1987).

1.14.1 *The clinical use of mitozantrone*

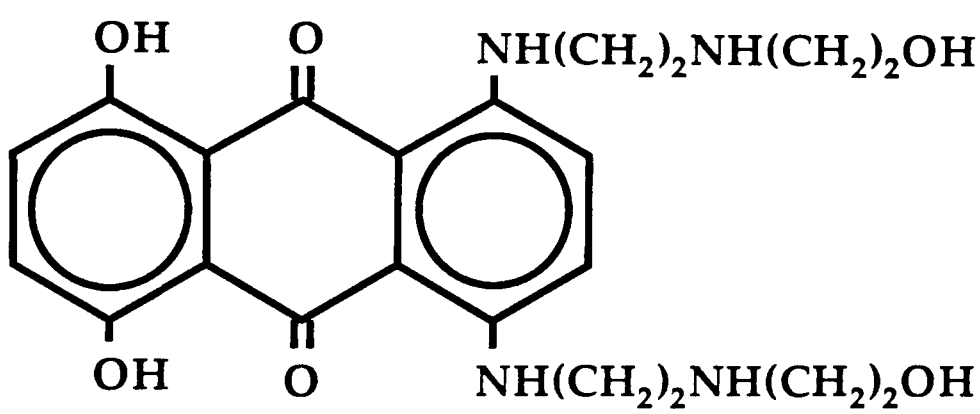


Figure 1.32. The structure of mitozantrone

Mitozantrone has produced a 33% response rate as a first line agent in advanced breast cancer (Saleton, 1987, Bennett *et al.*, 1988), a 9-64% response rate in patients with acute refractory leukaemia (Hiddemann, 1987) and up to a 43% response rate in acute lymphocytic leukaemia (reviewed by Shenkenberg and Von Hoff, 1986). It has also been successfully used in acute non-lymphocytic leukaemia (Vorobiof *et al.*, 1987), in treatment of lymphoma (Hansen *et al.*, 1988) and in epithelial ovarian cancer (Lawton *et al.*, 1987). Reviews on recent progress with mitozantrone therapy are given by Schenkenberg and Von Hoff (1986) and Bonadonna (1987).

1.15 The development of anthrapyrazole antitumour agents

Mitozantrone has a reduced cardiotoxicity but a reduced spectrum of activity compared to doxorubicin. In attempts to produce a non-cardiotoxic anthraquinone with a wider spectrum of activity the anthrapyrazoles have been developed (Leopold *et al.*, 1985; Showalter *et al.*, 1987). The cardiotoxicity induced by anthracyclines and anthraquinones has been associated with oxygen free radical generation [see section 1.16.4]. The rationale behind the development of the anthrapyrazoles was to render the electron deficient quinone chromophore of anthracyclines and anthraquinones more resistant to enzymic reduction whilst retaining the planar chromophore and basic side chains required for antitumour activity (Showalter *et al.*, 1986). The quinone moiety in the anthraquinone chromophore was modified to a quasi iminoquinone with an extra ring incorporated. CI941 (see figure 1.33) the most active of the anthrapyrazoles is currently undergoing phase I and II clinical trials.

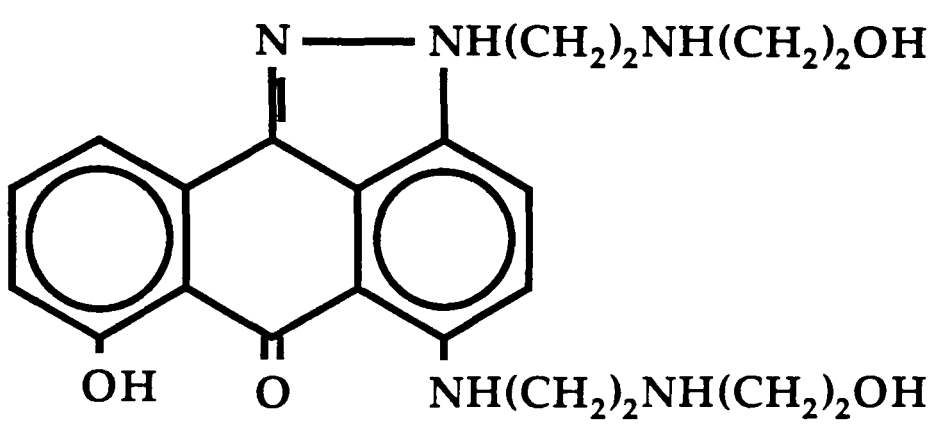


Figure. 1.33. Structure of CI941

1.16 The mechanism of action of anthracycline antitumour agents

The mechanisms of action of the anthracyclines will be discussed mainly with reference to doxorubicin [figure 1.29] as this is the most investigated and most clinically useful of this class of agents to date. Despite being categorised into distinct divisions there is considerable overlap between these mechanisms.

1.16.1 *DNA binding*

The anthracyclines doxorubicin and daunorubicin [figure 1.29] are taken up by cells and rapidly locate in the nucleus. These drugs have been shown to bind to DNA with physico-chemical characteristics consistent with intercalation [Lown, 1983; Aubel-Sadron *et al.* 1984; Arcamone 1984] [see section 1.6.2.1]. The anthraquinone chromophore is inserted between adjacent base pairs with reciprocal overlap and the amino sugar is situated in the major or minor groove with its charged amino group close to the second phosphate anion away from the intercalation site. The chromophore lies perpendicular to the base pair hydrogen bonds, ring A projects through the intercalation site, rings B and C overlap with adjacent base pairs and ring D projects through the intercalation site opposite to ring A (reviewed by Gianni *et al.*, 1983). The chromophore forms hydrogen bonds between the hydroxyl groups and the DNA phosphate groups or the nitrogen atoms of the DNA bases [Wang, 1987). The amino sugar has been found to be essential for stable DNA complex formation. A negative cooperativity model has been proposed for anthracycline intercalation in which binding of one drug molecule alters the conformation of the neighbouring binding site, preventing drug binding [reviewed by Gianni *et al.*, 1983]. At high drug:nucleotide ratios an alternative mode of binding is seen in which the amino sugar is bound by electrostatic interaction to the phosphate groups of the DNA backbone. This external binding results in the chromophore being available for other interactions such as iron binding or DNA crosslinking. DNA binding is associated with formation of a highly

condensed and compacted DNA structure and an overall thickening of the DNA fibre in isolated chromatin (Waldes and Center, 1982). The intercalation model for doxorubicin refers to isolated DNA. The situation *in vivo* is likely to be far more complex and will depend on the conformation of DNA [see section 1.3], presence of binding proteins and extent of methylation [see section 1.3.1]. Doxorubicin shows weaker affinity for isolated nucleosomal DNA with a higher affinity for linking regions (Fritsche *et al.*, 1987; Skalka *et al.*, 1982).
Daunorubicin shows preference for the bent DNA helices found in nucleosomes and unfolds the nucleosome to some degree (Pearlman and Simpkins, 1985).

The base specificity of the anthracyclines has been extensively investigated but no consensus of opinion has been reached (reviewed by Gianni *et al.*, 1983). Generally there has been good correlation between the affinity for DNA of anthracycline derivatives and their cytotoxicity (Molinier-Jumel *et al* ., 1978), however the poor DNA binder N-trifluoroacetyl adriamycin-14-valerate (AD32) is an exception to this rule (reviewed by Gianni *et al.*, 1983).

DNA binding has been found to be associated with inhibition of DNA and RNA synthesis [Kriebardis *et al.*, 1987] and by inhibition of template activity of DNA and RNA polymerase [Chuang *et al.*, 1987]. However no correlation has generally been found between inhibition of DNA synthesis and cytotoxicity of doxorubicin (Siegfried *et al.*, 1983). Furthermore, the doxorubicin derivative AD32 inhibits DNA and RNA synthesis but does not bind with high affinity to DNA, suggesting DNA binding is not critical in this inhibition (Israel, *et al.*, 1987).

1.16.2 *Membrane binding*

The cell membrane is the first barrier encountered by anthracyclines, hence membrane effects have been extensively studied. Anthracyclines bind avidly to cellular membranes due to their amphipathic nature.

Doxorubicin has been found to bind to the negatively charged phospholipids cardiolipin (Goormaghtigh *et al.*, 1980) and phosphatidyl serine (reviewed by Gianni *et al.*, 1983). Cardiolipin is an important constituent of the inner mitochondrial membrane which is involved in enzyme function and is found extensively in heart mitochondria and tumour cell membranes. Tritton, *et al.* (1983) propose that this offers an explanation for anti-tumour and cardiotoxic effects, as the affinity of doxorubicin for cardiolipin is similar to that for DNA. Binding to cardiolipin occurs via the protonated amino group on the daunosamine moiety (Goormaghtigh *et al.*, 1987).

An alternative mechanism of binding for uncharged doxorubicin is by partition into the hydrophobic areas of the membrane. Burke *et al.* (1988) propose the existence of a low affinity binding site involving surface binding via electrostatic interaction of amino group to cardiolipin and a high affinity site with the amino group bound as above but the aglycone hydrophobically associated with the lipid bilayer.

Doxorubicin has been found to change the surface charge properties of membranes (Kessel, 1979), to perturb lipid structure and fluidity of membranes in euoxic (Deliconstantinos, 1987) and hypoxic (Siegfried *et al.*, 1983) cells and to alter the permeability of the mitochondrial membrane (Croce *et al.*, 1986).

Structural perturbation by doxorubicin is associated with effects on membrane components. Doxorubicin inhibits cytochrome c oxidase, coenzyme Q dependent NADH dehydrogenase and succinate dehydrogenase in mitochondrial membranes (Goormaghtigh and Ruysschaert, 1984). In addition, doxorubicin and its metabolite doxorubicinol inhibit the Na/K pump of cardiac sarcolemma, the calcium pump in heart muscle (Boucek, *et al.*, 1987) and Na/K ATPase in heart microsomes (Gosalvez *et al.*, 1979, Miwa *et al.*, 1986). A specific calcium binding protein has also been proposed as a doxorubicin binding site (Canada *et al.*, 1988). Doxorubicin also inhibits oxidative phosphorylation, decreases ATPase activity and affects the redox state of redox carriers in tumour, liver

and heart mitochondria (Bianchi *et al.*, 1987). These effects suggest a role for membrane effects in cytotoxicity and cardiotoxicity.

Several workers have shown that doxorubicin, immobilised on microspheres, is more cytotoxic than free drug without entering cells [Tritton *et al.*, 1983; Rogers *et al.*, 1983; Wingard *et al.*, 1985]. Moreover, recently agarose bound doxorubicin has been found to be active *in vivo* and *in vitro* (Hacker *et al.*, 1989). This suggests membrane effects are important in this system. The amino group was not available for interaction with the membrane in this system, however the aglycone was available for iron binding or redox cycling. Alterations in membrane properties have also been shown to be intimately involved in resistance of cells to doxorubicin (Montaudon *et al.*, 1986; Marsh and Center, 1987).

1.16.3 *Inhibition of DNA topoisomerase II*

Topoisomerase II is a nuclear enzyme that is involved in control of the topology of DNA [see section 1.7.1]. Topoisomerase activity is associated with the proliferative status of the cell (Sullivan *et al.*, 1987; Markovits *et al.*, 1987). Doxorubicin was first noticed to inhibit this enzyme when single and double strand breaks mediated by doxorubicin in L1210 cells were shown to be associated with covalently bound protein molecules at the 3′ and 5′ termini of the breaks (Zwelling *et al.*, 1981). Doxorubicin was found to produce double strand breaks in end labelled plasmid pBR322 DNA in the presence of purified topoisomerase II and inhibit the strand passing activity of the enzyme in the P4 DNA unknotting activity (Tewey *et al.*, 1984). Strand breaks occur due to doxorubicin 'trapping' a cleavable enzyme complex, which after denaturation with strong protein denaturants reveals double strand breaks [see section 1.7.2].

Doxorubicin has been shown to produce mainly protein associated strand breaks in many cell lines including human colon adenocarcinoma cells

(Johnston *et al.*, 1983; Broggini *et al.*, 1988), rat mammary tumour cells (Evans *et al.*, 1986), P388 leukaemia cells (Capranico *et al.*, 1986; Maniar *et al.*, 1988), human lung fibroblasts and V79 chinese hamster lung cells (Lawrence, 1988) and human leukaemic cells (Mcgrath *et al.*, 1989).

Topoisomerase II has also been shown to participate in resistance of tumour cells to doxorubicin. Potmesil *et al.* (1988) found human leukaemic and normal lymphocytes were resistant to DNA cleavage by doxorubicin due to possession of low topoisomerase II activity. A similar finding was made by Deffie *et al.* (1989) who found a direct correlation between topoisomerase activity and doxorubicin cytotoxicity in doxorubicin sensitive and resistant P388 leukaemic cells. Glisson *et al.* (1986) found an epipodophyllotoxin [a topoisomerase II inhibitor] resistant cell line to be cross resistant to doxorubicin. In addition Davies *et al.* (1988) found doxorubicin hypersensitive cells were also sensitive to the topoisomerase II inhibitors VP16 and mAMSA.

Clinical evidence for a role of topoisomerase II in cytotoxic action of doxorubicin includes the resistance to doxorubicin of tumours such as B cell chronic lymphocytic leukaemia which express a low level of topoisomerase activity and the sensitivity of other cancers to doxorubicin such as lymphoma and acute lymphoblastic leukaemia which express high levels of topoisomerase. In epipodophyllotoxin and anthracycline hypersensitive cells that express high topoisomerase II activity, doxorubicin produced more strand breaks than in normal cells (Robson *et al.*, 1987).

1.16.4 *Free radical formatiom*

1.16.4.1 *Formation of free radicals by enzymic reduction of anthracyclines*

As described in section 1.8.2 antitumour quinones including anthracyclines can undergo one or two electron reduction by flavin reductase enzymes. Doxorubicin has been found to be one electron reduced to form a semiquinone free radical (Sato *et al.*, 1977, Kalyanaraman *et al.*, 1980). The semiquinone autoxidises in the presence of molecular oxygen [rate constant $4.4x10^{7}M^{-1}S^{-1}$] resulting in formation of superoxide anion and consumption of oxygen. This redox cycling process has been found to occur in rat (Kalyanaraman *et al.*, 1980) and human liver microsomes (Basra *et al.*, 1985), in the presence of reducing agents such as sodium borohydride (Lown and Chen, 1981) and in purified enzyme systems including NADPH-cytochrome P450 reductase (Bachur *et al.*, 1979) and xanthine oxidase (Schreiber *et al.*, 1987). Additionally, semiquinone formation and superoxide generation was detected in heart tissues (Thayer, 1977). Other enzymes that can carry out the reduction of doxorubicin include nitrate reductase, NADPH-cytochrome c reductase and lipoamide dehydrogenase (see section 1.8.2). A general mechanism for the mechanism of quinone activation was proposed by Bachur *et al.* (1978), in which superoxide anion formation leads to a damaging free radical cascade involving iron, hydrogen peroxide and hydroxyl radical production [see section 1.8.3] resulting in cell damaging processes such as DNA damage and lipid peroxidation [section 1.9].

The enzymes required for reductive activation of doxorubicin have been found in the nuclear envelope, proximal to the DNA a possible site for free radical damage (Bachur, 1982, Romano *et al.*, 1982). Sinha and Chignall (1979) and Sinha and Gregory (1986) found that one and two electron products of doxorubicin and daunorubicin were covalently bound to DNA. Addition of DNA to semiquinone generating system results in a quenching of the esr signal, indicating that the drug can not be reduced when bound to DNA (Peters *et al.*, 1986). The semiquinone has also been found to bind covalently to BSA and microsomal protein in a system of liver and heart NADPH-microsomes (Scheulen *et al.*, 1982).

Further evidence for an involvement of reactive oxygen species in doxorubicin cell kill comes from investigations on the effect of glutathione depleting agents on doxorubicin cytotoxicity. These agents

[eg buthionine sulphoxamine] have been found to enhance doxorubicin cytotoxicity in EHp3 carcinoma cells (Lee *et al.*, 1988), A2780 ovarian cancer cells (Hamilton *et al.*, 1985) and rat hepatocytes (Lavelle and Patterson, 1987). Furthermore, Russo and Mitchell (1985) showed that doxorubicin was preferentially toxic to V79 cells with depleted glutathione levels compared to cells with elevated glutathione levels. GSH levels were found to be reduced in doxorubicin treated cells indicative of doxorubicin induced oxidative stress (Meijer *et al.*, 1987), including HeLa cells (Al-Kabban *et al.*, 1988). Additionally, exogenous glutathione reductase protected hepatocytes from oxidative challege by doxorubicin (Babson *et al.*, 1981). The removal of GSH reduces the capacity of glutathione peroxidase to scavenge hydrogen peroxide and other peroxides produced by doxorubicin redox cycling. Bozzi *et al.* (1981) showed that daunomycin cytotoxicity was related to the ability of the cells to detoxify hydrogen peroxide by glutathione dependent mechanisms. A role for oxygen in doxorubicin mediated cell kill is further supported by doxorubicin being selectively toxic to well oxygenated tumour cells (Tannock *et al.*, 1982) and less toxic in hypoxic tumour cells (Smith *et al.*, 1980).

A role for iron in the cytotoxicity of doxorubicin, possibly via the Haber-Weiss reaction (section 1.8.3), is supported by the partial inhibition of doxorubicin mediated MCF-7 cell kill by membrane permeable iron chelating agents (Doroshow, 1986a).

Further evidence for reductive activation and redox cycling by doxorubicin comes from studies on lipid peroxidation and DNA strand breakage.

1.16.4.2 *Lipid peroxidation*

Polyunsaturated fatty acids in membranes are a vulnerable target for attack by oxygen radicals [section 1.9.1]. Lipid peroxidation produced by doxorubicin could account for many of the effects associated with membrane binding [section 1.16.2]. Doxorubicin has been shown to stimulate NADPH dependent lipid peroxidation in mouse heart and liver microsomes whilst oxygen radical scavengers and iron chelators diminish this activity (Mimnaugh *et al.*, 1983), in rat liver and heart mitochondria and microsomes (Griffin-Green *et al.*, 1988, Mimnaugh *et al.*, 1982) and isolated rat liver nuclei (Mimnaugh *et al.*, 1985). Lipid peroxidation products from nuclear membranes may also participate in DNA damage (Vaca, 1988).

It has been proposed that lipid peroxidation is more important in doxorubicin-mediated cardiotoxicity than antitumour activity as alpha-tocopherol blocked cardiotoxicity in mice but did not alter cytotoxicity in mouse P388 ascites tumour (Myers, 1977). Mimnaugh *et al.* (1982) found a direct correlation between stimulation of heart and liver microsomal lipid peroxidation and the cardiotoxic effects of anthracycline derivatives in animal models. Doxorubicin also produced increased lipid peroxidation in mouse heart *in vivo* (Patterson *et al.*, 1983).

1.16.4.3 *DNA strand breakage*

The majority of evidence for doxorubicin mediated DNA strand breakage comes from plasmid DNA strand breakage assays. In a system of cytochrome P450 reductase, NADPH and end labelled DNA , doxorubicin mediated strand cleavage in double and single stranded DNA, demonstrating that intercalation was not an essential requirement for strand breakage. Additionally in the absence of oxygen no strand breakage occurred (Berlin and Haseltine, 1981).

Lown *et al.* (1982) found a correlation between stimulation of microsomal oxygen consumption and DNA nicking in a system of doxorubicin, plasmid DNA and sodium borohydride. Sugar modified anthracyclines which were less easy to reduce and produced less strand breakage in this assay had reduced cardiotoxicity (Lown *et al.*, 1982). Similarly, in this assay the less cardiotoxic anthracycline 5-iminodaunorubicin produced less strand breakage (Lown *et al.*, 1979).

Doxorubicin and daunorubicin were found to bind to PM2 supercoiled DNA and induce nicks in the presence of sodium borohydride. This process was inhibited by SOD, catalase and sodium benzoate indicating involvement of superoxide anions, hydrogen peroxide and hydroxyl radicals [Lown *et al.*, 1977]. A similar result was found by Mong *et al.* (1980), drug alone causing conformational changes but no nicking in PM2 DNA. In the presence of sodium borohydride, NADPH cytochrome P450 reductase or NADH dehydrogenase doxorubicin nicked supercoiled PM2 DNA (Haidle and Hunter Mckinney, 1985 and 1986). Xanthine oxidase was found to reduce doxorubicin to a semiquinone which reacted with hydrogen peroxide to produce DNA strand scission under anaerobic conditions. This process was inhibited by superoxide dismutase (Bates and Winterbourn, 1982).

Doxorubicin has been shown to cause non-protein associated DNA strand breakage [see section 1.9.2] in some cellular systems. This non-protein associated strand breakage is possibly related to oxygen radical formation whereas protein associated strand breaks are related to inhibition of topoisomerase II activity [see section 1.7.2]. Goldenberg *et al.* (1986) showed that the cytotoxic action of doxorubicin was closely associated with double strand break formation in P388 leukaemia cells. Resistant cells had a lower level of single and double strand breaks than sensitive cells. In mouse leukaemic L1210 cells treatment with low doxorubicin concentrations produced protein associated strand break formation whereas treatment with high concentrations produced non-protein associated strand breaks (Potmesil *et al.*, 1984). Ross and Smith (1982) also found doxorubicin to cause single and double strand breaks and DNA protein crosslinks in L1210 cells. Pommier *et al.* (1983)

investigated the effect of oxygen radical scavengers on doxorubicin induced strand breakage in L1210 cells. DMSO and thiourea inhibited strand breakage by ionising radiation. In contrast, thiourea inhibited single strand break formation and DMSO stimulated strand break formation by doxorubicin. These effects were proposed to be due to different effects on DNA conformation. Non-protein associated strand breaks could be detected in doxorubicin treated V79 chinese hamster cells [Iliakis and Lazar, 1987] and daunorubicin treated rat hepatocytes and mammary epithelial cells (Capranico *et al.*, 1989). However, Capranico *et al.* (1986) found no correlation between redox potentials of sugar-modified anthracyclines and DNA strand breakage in P388 leukaemia cells. Similarly Potmesil *et al.* (1983) showed equivalent strand breakage by doxorubicin and the redox-inactive 5-iminodaunorubicin.

1.16.4.4 *The role of doxorubicin free radical formation in cellular resistance*

The development of drug resistance by tumour cells is a major problem in cancer treatment. To investigate the mechanisms involved, resistant cell lines have been characterised *in vitro*. The results of these studies have contributed indirect evidence for doxorubicin free radical involvement in cell kill. Mungikar *et al*. (1981) showed a decreased level of mixed function oxidase enzymes in doxorubicin-resistant P388 cells, particularly cytochrome b_5 and cytochrome P450. The resistant cells were also resistant to ascorbate induced lipid peroxidation. These results indicate a possible role for MFO activation and lipid peroxidation in the action of doxorubicin. Doxorubicin resistance has been associated in many cell lines with an increase in glutathione dependent enzymes. Glutathione-S-transferases are a group of enzymes involved in the detoxification of xenobiotics. Additionally, this enzyme has peroxidase activity [see section 1.10.1]. Glutathione-S-transferase activity was increased in mouse tumour cells (Dahllof *et al*., 1987) and in P388 leukaemic cells (Deffie *et al*., 1988) relative to sensitive cells. Batist *et al*. (1986) found that doxorubicin resistant MCF-7 human breast cancer cells overexpressed glutathione-S-transferase and glutathione peroxidase. In doxorubicin resistant MCF-7 cells an increase in phase I (cytochrome P450) and phase II metabolising enzymes (glutathione-S-transferase and glucuronyl transferase) was observed by Cowan *et al*. (1986). Depletion of glutathione in doxorubicin resistant MCF-7 human breast cancer cells using buthionine sulphoxamine [BSO], an inhibitor of GSH synthesis, potentiated doxorubicin mediated hydroxyl radical formation and cytotoxicity.

In contrast to the above studies, Yusa *et al*. (1988) did not indicate that increase in glutathione S-transferase altered the sensitivity of Hattori and MCF-7 human breast cancer cells. Furthermore, Capranico *et al*. (1986) found no increase in cytotoxicity or DNA strand break formation by doxorubicin in glutathione depleted cell lines. In addition, Ramu *et al*. (1984) found no significant difference in the levels of enzymic detoxification systems in P388 murine leukaemia cells,

sensitive and resistant to doxorubicin.

Yeh *et al.* (1987) found reduced activity of the pentose phosphate shunt in doxorubicin resistant MCF-7 cells. This pathway provides NADPH for the maintenance of reduced glutathione via glutathione reductase and for flavin based oxidoreductases. These authors suggest reduced activity of the pentose phosphate shunt may limit cytochrome P450 reductase mediated doxorubicin activation [see section 1.8.2] in the resistant cells. Mimnaugh *et al.* (1989) showed that doxorubicin -resistant MCF-7 cells were cross resistant to other oxygen free radical generating systems [eg xanthine/xanthine oxidase and hydrogen peroxide]. Hyperoxia was found to potentiate cytotoxicity in resistant and sensitive cells. Resistant cells also had increased SOD and glutathione peroxidase activities. In a doxorubicin resistant human small cell lung cancer line cross resistant to radiation, an increased rate of DNA repair was suggested to play a role in the resistant mechanism (Meijer *et al.*, 1987). Lower reduced glutathione pools in doxorubicin treated sensitive cells were indicative of drug induced oxidative stress (Meijer *et al.*, 1987).

1.16.4.5 *Free radical formation by anthracycline-metal complexes*

Hydroxyquinones have been shown to form metal complexes with the metal ion coordinated by the quinone carbonyls and adjacent hydroxyl groups to form a six membered chelate (reviewed by Gianni *et al.*, 1983). Doxorubicin has been to shown to form complexes with a variety of metal ions (reviewed by Garnier-Suillerot, 1988), this chelation giving rise to an intramolecular charge transfer to the hydroxyanthraquinone moiety (Myers *et al*, 1982). Doxorubicin forms a 1:3 complex with iron (III) (Beraldo, 1985) which has a similar stability constant to the 1:1 complex of iron (III) with desferrioxamine (reviewed by Gianni *et al.*, 1983). Doxorubicin has also been shown to form 1:1 and 1:2 complexes with Cu (II). Both Fe and Cu been proposed to be involved in binding of doxorubicin to DNA [Eliot *et al.*, 1982; Spinelli and Dabrowiak, 1982]. This occurs via formation of a doxorubicin-Fe-DNA ternary complex involving a mordant bond (reviewed by Garnier-Suillerot, 1988).

Doxorubicin-Fe^{3+} has also been shown to catalyse transfer of electrons from glutathione to oxygen (Myers *et al.*, 1982). Since this complex can bind to membrane cardiolipin (Hasinoff *et al.*, 1989) and DNA (reviewed by Garnier-Suillerot, 1988) this mechanism is possibly involved in membrane lipid damage and DNA strand breakage. Indeed a system of glutathione and doxorubicin-iron complex caused DNA cleavage (Eliot *et al.*, 1984; Muindi *et al.*, 1985) and destruction of erythrocyte membranes (Myers *et al.*, 1982) by mechanisms involving oxygen free radicals. In addition, Muindi *et al.* (1984) showed that doxorubicin-Fe(III) produced hydroxyl radicals in the presence of hydrogen peroxide which resulted in DNA strand cleavage. Doxorubicin-iron or -copper complexes have also been shown to inactivate cytochrome c oxidase (Hasinoff *et al.*, 1989) and protein Kinase C (Hannun *et al.*, 1989).

Doxorubicin can also form an doxorubicin-iron-ADP complex which can take place in the initiation of lipid peroxidation. It is thought that this and the doxorubicin-iron complex can redox cycle due to reduction by cytochrome P450 reductase leading to production of superoxide anions, hydrogen peroxide and hydroxyl radicals (Gianni *et al.*, 1985). However daunorubicin and doxorubicin iron complexes were not found to redox cycle in the presence of NADH dehydrogenase (Beraldo *et al.*, 1985). It has also been proposed that the reduction of drug-bound iron is brought about by oxidation of the Ketoaldehyde group on C_9 of doxorubicin (Gianni *et al.*, 1985). Once reduced the doxorubicin-Fe(II) complex can catalyse hydroxyl radical formation via the iron catalysed Haber-Weiss reaction [see section 1.8.3]. Gianni *et al.* (1988) found that oxygen free radicals were not involved in initiation of lipid peroxidation by the doxorubicin-iron complex. This is in agreement with the mechanism of initiation proposed by Minotti and Aust, 1987 [see section 1.9.1]. It may not always be possible to distinguish between drug redox cycling and drug-bound iron redox cycling in a particular system as iron is a ubiquitous contaminant of laboratory reagents.

1.16.4.6 *Bioreductive alkylation by doxorubicin*

Under anaerobic conditions the formation of the doxorubicin semiquinone by enzymic reduction has been proposed to result in the formation of an alkylating species (reviewed by Gianni *et al.*, 1983). The semiquinone undergoes a rearrangement in which the unpaired electron migrates to the C_7 position with resulting cleavage of daunosamine [see figure 1.34]. This leaves a C_7 aglycone radical which is proposed to be an alkylating species. It is this radical that is thought to be involved in covalent binding of doxorubicin to DNA (Sinha and Chignall, 1979). If this free radical abstracts a hydrogen atom the product is a 7-deoxyaglycone. The 7-deoxyglycone of daunorubicin has been shown to be metabolically reduced (Oldcorne and Patterson, 1987).

An alternative proposal for formation of a doxorubicin alkylating species involves the formation of a two electron reduction product the hydroquinone (Moore, 1977; Moore and Czerniak, 1981). Formation of the hydroquinone results in the aminosugar becoming a stable leaving group. The loss of the C_7 aminosugar results in the formation of a quinone methide intermediate. This is envisaged to act as a nucleophile to give a C_7 functionalised quinone or as an electrophile to give a C_7 functionalised hydroquinone [see figure 1.34]. This latter product may re-form the quinone methide unless oxidised to the more stable quinone state. Alternatively the quinone methide will react with a hydrogen atom to form the 7-deoxyaglycone or with itself to form a 7-7'-dimer (reviewed by Gianni *et al.*, 1983; Powis, 1989). However, there is no direct evidence to suggest doxorubicin can undergo this mechanism since no doxorubicin dimers have been isolated.

Either pathway could lead to the alkylation of critical cellular macromolecules including membranes and DNA. These mechanisms could be particularly prevelant in the hypoxic areas of tumours (reviewed by Kennedy *et al.*, 1980) and have been proposed to be involved in the cardiotoxic effects of doxorubicin (Cummings *et al.*, 1987).

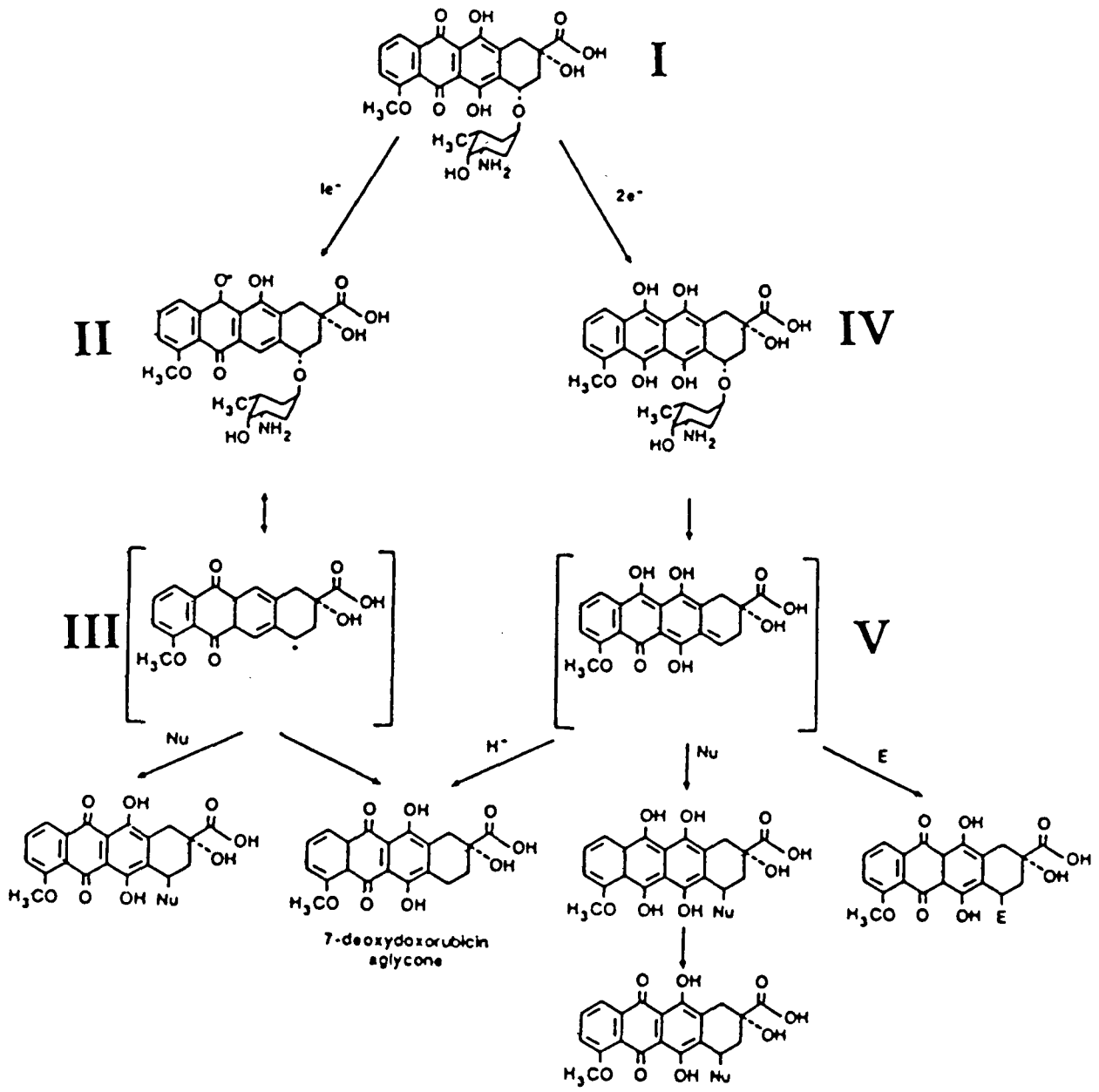


Figure 1.34. *Pathways for formation of alkylating species from doxorubicin (I) by one electron reduction to the semiquinone radical (II) and a postulated C-7 carbon centred radical (III), or by two electron reduction to the hydroquinone (IV) and a postulated quinone-methide (V). Nu is a nucleophile, E is an electrophile.*

(Powis, 1989)

1.16.5 *Mechanisms of doxorubicin cardiotoxicity*

As described previously the clinical use of doxorubicin has been associated with a dose limiting cardiotoxicity (reviewed by Unverferth *et al.*, 1982; Sinha, 1982). The cardiotoxicity of doxorubicin can be divided into acute effects and chronic effects. Acute cardiovascular effects include hypotension, tachycardia and various arrhythmias. The chronic effect is cardiomyopathy leading to congestive heart failure (reviewed by Singal, 1987). Thirty five percent of patients treated with a cumulative dose greater than 600 mg/m^2 experienced chronic effects. Morphological changes in the myocardium include dilation of myocyte sarcoplasmic reticulum, dissolution of contractile protein and changes in mitochondria (reviewed by Gianni *et al.*, 1983).

The consensus of opinion is that reductive activation of doxorubicin with concomitant formation of oxygen free radicals in heart tissue leads to the cardiotoxicity observed. Myocardial cells have been found to have a limited capacity to detoxify oxygen free radicals due to low levels of SOD and catalase. In addition doxorubicin has been found to suppress glutathione peroxidase activity in heart tissue (Doroshow *et al.*, 1980). Doxorubicin has been shown to be capable of redox cycling in cardiac sarcosomes (Doroshow and Reeves, 1981), rat heart subcellular fractions (Doroshow, 1983), cardiac sarcoplasmic reticulum and submitochondrial particles (Doroshow and Davies, 1983), heart mitochondria and microsomes (Nohl and Jordan, 1983) and cardiac myocytes (Doroshow, 1987). The doxorubicin metabolite doxorubicinol has also been shown to redox cycle in cardiac tissue (Gervasi *et al.*, 1986) and has been proposed to be involved in cardiotoxicity (Danesi, 1987). A heart specific mitochondrial NADH-oxidoreductase has been proposed to be the site of doxorubicin activation in the heart tissue (Nohl, 1988). Doxorubicin has also been found to deplete glutathione levels (Olson *et al.*, 1980) and initiate lipid peroxidation in heart tissue Lavelle *et al.*, 1985). Furthermore, Julicher (1985) found that rat heart cells were more sensitive to doxorubicin when glutathione levels were depleted. Further support of free radical involvement in cardiotoxicity are several studies which have found that anthracyclines shown to be

less cardiotoxic than doxorubicin are associated with reduced propensity for reductive activation. These include 5-iminodaunorubicin (reviewed by Gianni *et al.*, 1983), epirubicin (figure 1.30) [reviewed by Johnston and Glazer, 1984; Goodman and Laughner, 1986] and 4'-demethoxy-4'-4'-O-methylodoxorubicin (figure 1.31) [Arcamone, 1984). The substituent groups of the aglycone and daunosamine have been found to be critical in relation to redox cycling capability of anthracyclines and their relative cardiotoxicity (Lown *et al.*, 1982). In addition, Free radical scavengers such as alpha-tocopherol, cysteamine and N-acetyl cysteine have been shown to lessen the cardiotoxic effects of doxorubicin (Olson *et al.*, 1981). More recently, two components to doxorubicin cardiotoxicity have been proposed. These involve oxidative and non-oxidative mechanisms of inactivation of mitochondrial electron transport chain components, including NADH dehydrogenase, succinate dehydrogenase, succinate oxidase and cytochrome c oxidase (Marcillat *et al.*, 1989).

The doxorubicin-iron complex has also been proposed to be involved in doxorubicin cardiotoxicity [see section 1.16.4.5]. The EDTA derivative 1,2-bis-(3,5-dioxo-piperazinyl-1-yl)propane (ICRF-187) was found to protect against doxorubicin cardiotoxicity in rabbits (Herman and Ferrans, 1987) and is currently undergoing clinical trials as a protectant during doxorubicin therapy.

Cardiotoxicity has also been proposed to be due to interaction of doxorubicin with cardiolipin, an important lipid located in the mitochondrial membrane which is involved in the function of several enzymes [see section 1.16.2]. Association constants of a series of anthracyclines with cardiolipin was found to correlate with their relative cardiotoxicity (Goormaghtigh *et al.*, 1980).

1.17 Mechanism of antitumour action of mitozantrone

As previously described (section 1.14) mitozantrone (figure 1.33) is an anthraquinone antitumour agent that was developed with a view to producing a less cardiotoxic agent than doxorubicin or daunorubicin (White and Durr, 1985). The mechanism of action of mitozantrone is reviewed by Durr *et al.* (1983) and Durr (1984). DNA is thought to be the major target of mitozantrone as has been shown for the anthracyclines [section 1.16.1]. Two binding sites have been proposed, a high affinity partial intercalation site and a lower affinity external electrostatic interaction with the sugar phosphates. The hydroxyethylamino side chains are proposed to partially preclude incorporation of mitozantrone into the double helix hydrophobic interior and hence part of the molecule lies in the minor groove (Foye *et al.*, 1982; Kapuskinski *et al.*, 1981; Lown *et al.*, 1984; Lown *et al.*, 1985; Krishnamoorthy, 1986; and Chen *et al.*, 1986). Mitozantrone has been proposed to bind mainly to specific DNA regions by a mechanism which involves nearest neighbour exclusion (Rosenberg *et al.*, 1986). It has also been proposed that mitozantrone binds by a mechanism that favours binding of other mitozantrone molecules (positive cooperativity) to some DNA regions (Rosenberg *et al.*, 1986). Mitozantrone molecules have been shown to stack when they are externally bound to DNA (Kapuscinski *et al.*, 1984). Mitozantrone DNA binding has been proposed to responsible for its potent inhibition of DNA and RNA synthesis which has been found in mouse L cells (Nishio and Uyeki, 1983), T47D breast tumour cells (Safa *et al.*, 1983) and MDA-MB-231 breast tumour cells (Chegini and Safa, 1987). Furthermore mitozantrone has been shown to inhibit RNA polymerase (Foye *et al.*, 1982). Mitozantrone has also been shown to condense nucleic acids (particularly RNA) [Kapuscinski and Darzynkiewicz, 1984 and 1986].

As described in section 1.16.5 doxorubicin mediated cardiotoxicity has been associated with free radical generation (redox cycling). Consequently the free radical chemistry of the less cardiotoxic [see section 1.14] mitozantrone has been widely investigated. Mitozantrone has been found to produce cardiotoxicity in patients pretreated with

anthracyclines (reviewed by Shenkenberg and Von Hoff, 1986). Substantially less redox activity was found with mitozantrone in rabbit heart microsomes, purified NADPH cytochrome P450 reductase and NADH-dehydrogenase compared to doxorubicin (Kharasch and Novak, 1983a and 1985). This was also found in human liver microsomes (Basra *et al.*, 1985) and rat liver and heart microsomes (Basra, 1986). In addition Doroshow and Davies (1983) found mitozantrone did not significantly enhance oxygen metabolism in cardiac sarcoplasmic reticulum and submitochondrial particles. Mitozantrone inhibited base rate NADPH dependent lipid peroxidation in rabbit liver microsomes and rabbit cardiac sarcosomes, inhibited NADH dependent lipid peroxidation in cardiac mitochondria and inhibited iron stimulated lipid peroxidation in rabbit liver microsmes (Kharasch and Novak, 1983b and 1985). In addition mitozantrone did not initiate lipid peroxidation in mouse liver and heart fractions (Patterson *et al.*, 1983). Doxorubicin, in contrast, stimulated lipid peroxidation in all the above systems. Mitozantrone was also found to inhibit doxorubicin stimulated lipid peroxidation and, to a lesser extent, superoxide formation (Kharasch and Novak, 1982). Other effects of mitozantrone include inhibition of platelet aggregation and prostaglandin and thromboxane biosynthesis (Frank and Novak, 1986) and inhibition of tumour angiogenesis (Polverini and Novak, 1986).

The mechanism of action of anthraquinone antitumour agents such as doxorubicin and mitozantrone is clearly not fully understood. The absence of a systematic study on the role of reductase mediated redox cycling of these agents in their antitumour activity is strikingly apparent. Furthermore, more information is required on the relationships between cellular DNA damage by these agents, their propensity to redox cycle and their cytotoxicity. The next section sets out the aims of the present study.

1.18 Aims

(1) The suitability of the MCF-7 human breast cancer cells as a model cell line for the investigation of antitumour drug redox cycling will be investigated using doxorubicin as a reference system.

(2) The propensity to redox cycle by mitozantrone, CI941 and a series of alkylaminoanthraquinones (figure 1.35) based on mitozantrone will be investigated in the MCF-7 cell line. This will allow the relationship between side chain substitution pattern and redox cycling propensity to be assessed.

(3) The propensity of the above agents to mediate plasmid DNA strand breakage in the presence of purified reductase enzymes will be investigated. Doxorubicin will be used as a reference system. This will provide further information as to the relative propensities of these agents to redox cycle.

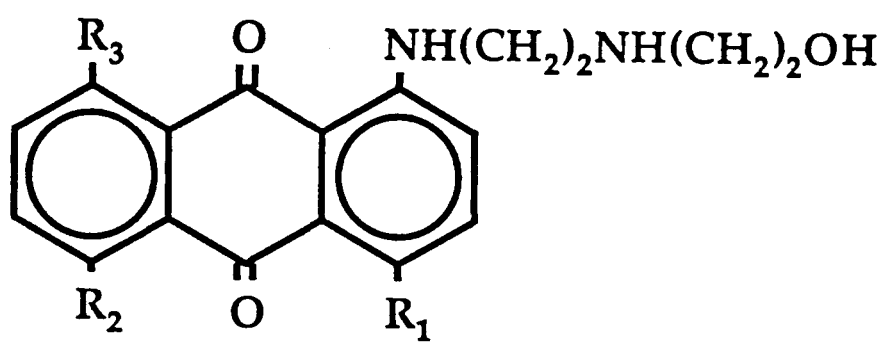
(4) DNA strand breakage produced by the above agents will be determined in the MCF-7 cell line. This will allow the relationship between DNA strand breakage and the propensity to redox cycle of these agents to be ascertained.

(5) The cytotoxicity of these agents will be determined in the MCF-7 cell line. Cytotoxicity of these agents will be related to their relative propensity to redox cycle and produce cellular DNA strand breakage in MCF-7 cells.

(6) Cellular uptake of the above agents will be determined in MCF-7 cells. This will determine whether differences in uptake can account for any differences in DNA strand breakage and cytotoxicity observed for these agents.

(7) The effect of the above agents on MCF-7 cell topoisomerase activity will be determined. This will reveal whether topoisomerase-DNA cleavable complex formation contributes to the cellular DNA strand breakage and cytotoxicity of these agents in MCF-7 cells.

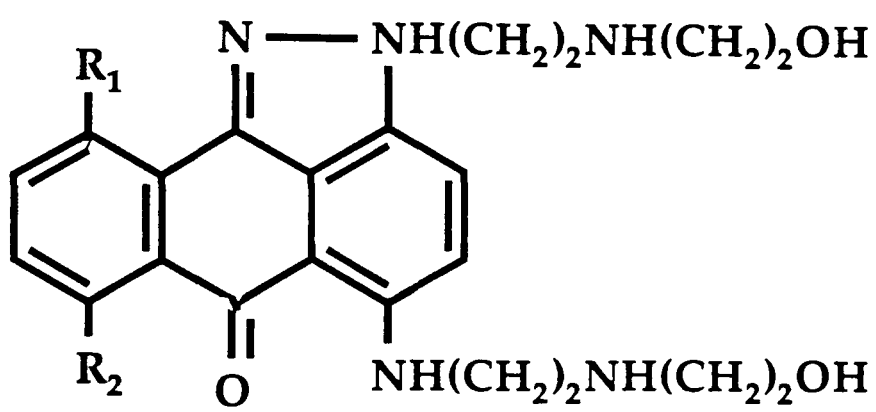
(8) The ability of the above agents and a series of anthrapyrazole antitumour agents based on CI941 (figure 1.36) to mediate plasmid DNA strand breakage in the presence of iron and reduced glutathione will be investigated. This will determine whether iron binding is a possible alternative mechanism for these agents.



AQ	R1	R2	R3
1AQ	H	H	H
1,4AQ	X	H	H
1,5AQ	H	X	H
1,8AQ	H	H	X

$X = NH(CH_2)_2NH(CH_2)_2OH$

Figure 1.35 The structure of alkylaminoanthraquinones



APZ	R1	R2
7,10-OH	OH	OH
7,10-H	H	H
10-OH	OH	H
7-OH (CI941)	H	OH

Figure 1.36. Structure of anthrapyrazoles

CHAPTER 2

An investigation of redox cycling by quinone antitumour agents in MCF-7 human breast cancer cell fractions

CHAPTER 2

An investigation of redox cycling by quinone antitumour agents in MCF-7 human breast cancer cell fractions

2.1 Introduction

As described in section 1.8.2 one of the proposed mechanisms of action of quinone containing antitumour agents is reductase mediated redox cycling, resulting in the production of cell damaging reactive oxygen species [see figure 1.19]. Section 1.8.3 shows the series of reactions which can result from superoxide anion formation. From figure 1.19 it can be seen that there are several lines of evidence which are indicative of drug redox cycling in a biological system:-

1. Oxidation of NADPH.
2. Formation of drug semiquinone free radicals.
3. Formation of superoxide anions.
4. Formation of hydroxyl radicals.

In this chapter redox cycling by the quinone antitumour agents of the present study is investigated in MCF-7 human breast cancer cells. This cell line is particularly relevant for these studies since doxorubicin and mitozantrone are used in the treatment of advanced breast cancer [see sections 1.13 and 1.14].

2.2 Methods

2.2.1 *Determination of the rate of NADPH oxidation in cellular S9 homogenates*

This was measured by following the decrease in NADPH absorbance at 340nm. All measurements were made using an uv/vis scanning spectrophotometer [552 spectrophotometer, Perkin Elmer] fitted with a temperature controlled Pelte cell [Perkin Elmer]. The reaction mixture [1ml], consisting of sodium phosphate buffer [180mM, pH 7.4], potassium chloride [225mM], nicotinamide [7.5mM] and MCF-7 S9 fraction [100ul, 0.2-0.4mg S9 protein] was incubated at 37C for 5 minutes before the reaction was initiated by the addition of NADPH [0.1mM]. NADPH oxidation was followed for 3 minutes to give a base rate. Then, an appropriate volume of drug was added to give the desired final concentration and the reaction followed for a further 3 minutes. The rate of NADPH oxidation was calculated using the extinction coefficient of 6.22mM cm^{-1} [Handa and Sato, 1976] at 340nm and the results expressed as nmole min^{-1} mg^{-1} protein.

2.2.2 *Determination of superoxide generation*

2.2.2.1 *Reduction of acetylated cytochrome C*

Superoxide generation was determined by following the reduction of acetylated cytochrome C at 550nm (see section 1.12.1). All measurements were carried out on the spectrophotometer described above. The reaction mixture consisted of potassium chloride [150mM]-Tris [50mM, pH7.4], acetylated cytochrome C [0.07mM] (see appendix A3.0 for details of preparation) and NADPH [1.0mM]. After prewarming to 37C for 5 minutes, the reaction was initiated by the addition of S9 fraction (25ul, 0.05-0.15mg S9 protein) and the reduction of acetylated cytochrome C followed

for 3 minutes. To determine the effect of drug on superoxide generation a separate incubation was set up with the appropriate concentration of drug. To establish that the reaction rate observed was due to superoxide generation the reaction was repeated in the presence of superoxide dismutase [300ug ml^{-1}] or bovine serum albumin [300ug ml^{-1}]. BSA was added to ensure that the superoxide dismutase inhibition observed was not due to addition of protein. The rate of acetylated cytochrome C reduction was determined using the extinction coefficient of 19.6mM cm^{-1} at 550nm [Azzi *et al.*, 1975]. The results were expressed as nmoles acetylated cytochrome C formed $min^{-1}mg^{-1}$protein.

2.2.2.2 *Formation of adrenochrome from adrenaline.*

This assay is dependent on the conversion of adrenaline to adrenochrome by superoxide and the measurement of adrenochrome formation (see section 1.12.1) spectrophotometrically at 480nm [as described in section 2.2.2.1]. The incubation [1ml] mixture consisted of potassium phosphate buffer [100mM, pH7.4], EDTA [1mM], adrenaline bitartrate [2mM in 0.02M HCl], NADPH [1.0mM] and an appropriate volume of drug to produce the concentration required. The above mixture was incubated at 37C for 3 minutes prior to the initiation of the reaction by addition of S9 fraction [50ul, 0.1-0.3mg S9 protein], the reaction being followed for 5 minutes. Appropriate controls were carried out in the absence of drug or S9 fraction. The reaction was repeated in the presence of superoxide dismutase [900 units, 560ug protein] or BSA [560ug] to ensure the observed adrenochrome formation was due to superoxide, since hydrogen peroxide can also mediate the conversion of adrenaline to adrenochrome. BSA was added for the reasons described in section 2.2.2.1.

The rate of adrenochrome formation was quantitated using an extinction coefficient of 4.02mM cm^{-1} at 480nM (Peterson *et al.*, 1979). The results were expressed as nmoles adrenochrome formed $min^{-1}mg^{-1}$protein.

2.2.3 *Determination of oxygen consumption in MCF-7 cells.*

Oxygen consumption was measured using a metabolic oxygen meter [SEA] with a polarizable Clark type electrode linked to a chart recorder [PM8251, Phillips]. The instrument was calibrated between 0 and 100% dissolved oxygen using sodium dithionite [10mg ml^{-1}] solution and air saturated distilled water respectively.

The reaction mixture [1ml] consisted of potassium chloride [150mM]-Tris [50mM, pH7.4] buffer, magnesium chloride [10mM], MCF-7 cells ($10^6 ml^{-1}$) and NADPH [0.1mM]. The above mixture was placed in the sample well [37C] of the oxygen meter and a magnetic stir bar and electrode inserted. Oxygen consumption was followed for 8 minutes. A separate incubation was set up with the required drug concentration and treated as above.

The rate of oxygen consumption was determined using the datum that 1ml distilled water at 25C contains 0.237 umoles of oxygen [Lessler, 1978].

2.2.4 *Investigation of drug semiquinone free radical formation using electron spin resonance spectrometry [esr]*

Esr spectrometry (see section 1.12.2) was carried out using a Varian E109 X-band esr spectrometer at room temperature [typically 20-23C]. Studies were carried out on samples loaded into a quartz flat cell [1.5ml].

The reaction mixture [1ml] consisted of potassium phosphate buffer [100mm, pH7.4], S9 fraction [1.7 mg protein] or MCF-7 cells [$3.5x10^5$], NADPH [0.1mM] and drug. The above mixture was placed in septum sealed crimped vial and deaerated for 15 minutes with oxygen-free nitrogen [BOC] by means of a fine cannula [inserted via a 19 gauge needle].

After deaeration the sample was taken up into a syringe [1ml] and injected into the top of the quartz flat cell which had been flushed with nitrogen. Prior to stoppering, the flat cell was again flushed with nitrogen.

The flat cell was clamped into the cavity and the instrument tuned. The required operating parameters were set on the instrument, typically microwave power 5mW, field set 3371 Gauss, field sweep +40 Gauss, time constant 1 second, modulation amplitude 4 Gauss, receiver gain 10^4 and scan time 4 minutes.

If an esr signal was detected 4 minute scans were repeated for 30 minutes to determine the intensity and duration of the spectrum. In some experiments the esr spectra were accumulated using a Nicolet data accumulator [model 1170, Instrument Corp] using ten, two minute scans.

2.2.5 *Determination of oxygen free radical formation in MCF-7 S9 fraction or MCF-7 cells by spin trapping*

The principles of this technique are fully described in section 1.12.2. The spin trap 5,5-dimethyl-1-pyrroline N-oxide [DMPO] was used in these studies. Esr was carried out as described in section 2.2.4.
Each incubate was made up of the following components in the order of addition:- Phosphate buffer [200mM, pH7.4] (bubbled with oxygen for five minutes), MCF-7 S9 fraction [0.68 mg protein ml^{-1}] or MCF-7 cells [$3.5x10^5$], Desferrioxamine [1mM], DMPO [100mM], NADPH [0.5mM] and drug [400uM].

To confirm that the spin adducts produced were drug and S9 fraction dependent the above incubate was repeated in the absence of S9 fraction or drug.

In order to confirm that the esr signal obtained was due to oxygen

radical formation, the above incubate was repeated in the presence of DMSO [200mM], catalase [300 units ml^{-1}] or SOD [1000 units].

Esr measurements were determined in a quartz flat cell with the upper stopper removed. Typically the following parameters were used:- Microwave power 10mW, microwave frequency 9.467 GHz, modulation amplitude 2.0G, time constant 0.25 seconds, scan time 4 minutes, field set 3381, scan range 100G, receiver gain $8x10^4$, temperature 20-23C. Esr data was accumulated on an Archimedes data acquisition system using a kinetic programme [Brivati, J., Leicester University]. This programme allowed storage of each spectrum recorded so changes in the signal could be detected.

2.3 Results

2.3.1 *Investigation of free radical formation by doxorubicin in MCF-7 cells*

MCF-7 S9 fractions were chosen for these and subsequent experiments because it has been shown previously that this cell line contains the necessary enzymology for reductive activation of doxorubicin including NADPH-cytochrome c reductase, NAD H-cytochrome b_5 reductase and xanthine oxidase (Sinha *et al.*, 1987). These enzymes have been shown to be present in S9 fraction which is composed of the cellular microsomal and cytosolic components (Beaune and Guengerich, 1988). This fraction is free from interference from drug-DNA interactions [doxorubicin and other quinone containing agents bind strongly to DNA (reviewed by Gianni *et al.*, 1983)] [see section 1.16]. Preparation of MCF-7 S9 fraction is described in appendix A7.0.

2.3.1.1 *Effect of doxorubicin on NADPH oxidation in MCF-7 S9 cell fraction.*

The effect of doxorubicin on NADPH oxidation in MCF-7 S9 fraction was investigated in order to determine whether doxorubicin was metabolised by reductase enzymes that require NADPH as a cofactor [section 1.8.2]. NADPH oxidation was determined as described in section 2.2.1.

It can be seen from figure 2.1 that doxorubicin stimulated base rate NADPH oxidation in a concentration dependent manner. Doxorubicin [100uM] produced a ten fold stimulation of the base rate.

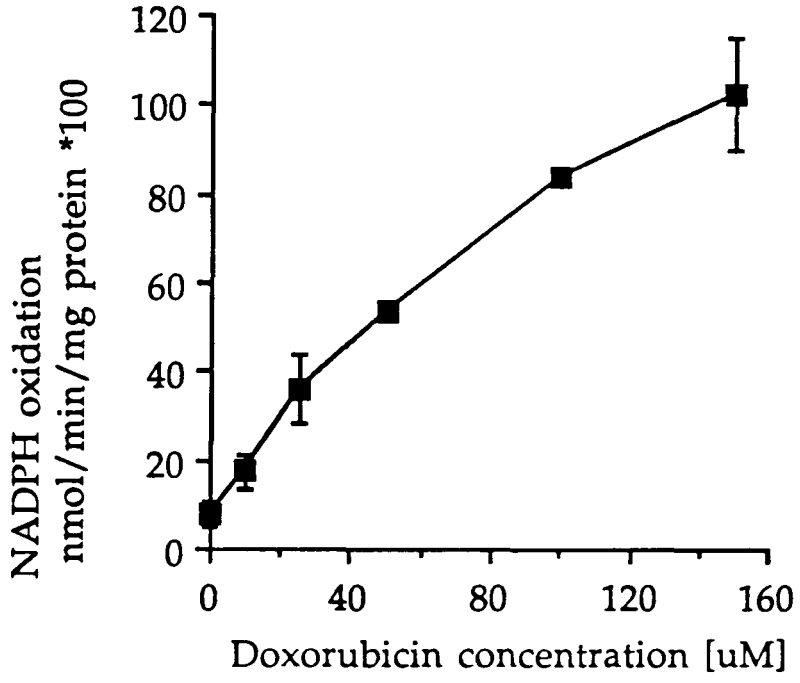


Figure 2.1 *The effect of doxorubicin on NADPH oxidation in MCF-7 S9 cell fraction.*

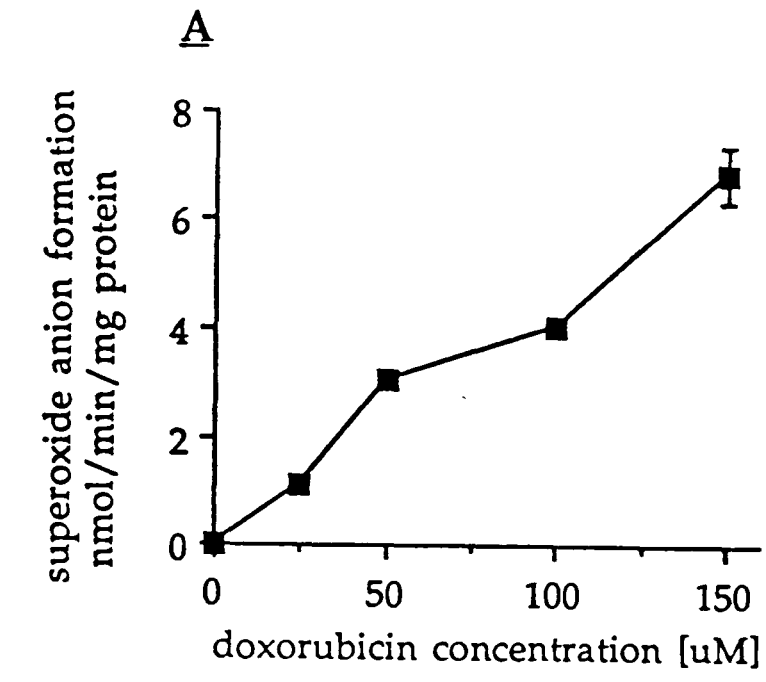
Base rate NADPH oxidation was 72 + 18 pmol $min^{-1}mg^{-1}$ protein.
Each data point is the mean + standard deviation of three determinations.

2.3.1.2 *Effect of doxorubicin on superoxide anion formation in MCF-7 S9 cell fraction.*

Having determined that doxorubicin stimulated NADPH oxidation in MCF-7 cell S9 fraction, which is evidence in part for reductive activation of the drug, the effect of doxorubicin on superoxide anion formation in this system was determined. Figure 2.2A shows doxorubicin stimulated superoxide anion formation as determined by reduction of acetylated cytochrome c in a concentration dependent manner. The effect of superoxide dismutase [SOD] on the rate of doxorubicin [100] stimulated acetylated cytochrome c reduction was determined. Doxorubicin [100uM] in the absence of SOD produced a rate of reduction of acetylated cytochrome c of 20.9 + 1.9 nmole min^{-1} mg^{-1} S9 protein whilst in presence of SOD (900 units) produced a rate of 13.36 + 0.84 nmole min^{-1} mg^{-1} S9 protein [base rate]. This demonstrated that 7.54 nmoles superoxide anion min^{-1} mg^{-1} S9 protein was produced by doxorubicin (100uM). When bovine serum albumin [BSA] was used instead of SOD (at an equivalent concentration) no inhibition of doxorubicin stimulation of acetylated cytochrome c reduction was seen. This demonstrated that the inhibition produced by SOD was not simply due to the addition of protein to the system.

Doxorubicin mediated superoxide formation in MCF-7 cell S9 fraction was also determined using the adrenochrome assay. Figure 2.2B shows SOD inhibitable adrenochrome formation in this system. It can be seen from this figure that doxorubicin stimulated superoxide anion formation in a concentration dependent manner. BSA had no effect on doxorubicin stimulated superoxide formation.

These results combined with those described in section 2.3.1 were consistent with doxorubicin undergoing reductive activation in a redox cycling process with resultant oxidation of NADPH and concomitant formation of superoxide anions. To determine whether these events were associated with formation of a doxorubicin semiquinone, the central species in the process of redox cycling, esr studies were carried out.



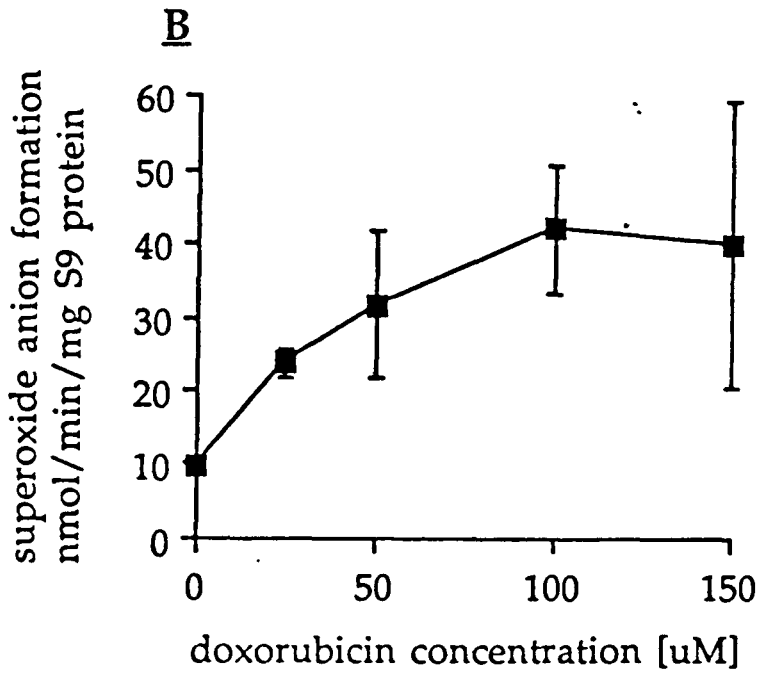


Figure 2.2 *Effect of doxorubicin on superoxide anion generation in MCF-7 S9 cell fraction as determined by SOD inhibitable reduction of acetylated cytochrome c [A] and adrenochrome formation [B].*

Data points are the mean + standard deviation of three determinations.

2.3.1.3 *Formation of doxorubicin free radicals in MCF-7 S9 cell fraction*

Figure 2.3 shows the first derivative esr spectrum observed upon incubation of doxorubicin [200uM] with NADPH fortified MCF-7 S9 fraction under anaerobic conditions. This signal had a peak to peak width of 4.2 Gauss and a g value of 2.00461, both parameters characteristic of an organic free radical (reviewed by Foster, 1984). No esr signal was obtained in the absence of drug, absence of S9 fraction or presence of air.

To further investigate doxorubicin free radical formation in MCF-7 cell S9 fraction, the effect of drug concentration on the esr signal intensity was investigated. Figure 2.4 shows that the intensity of the signal increased in a concentration dependent manner up to 300uM. A doxorubicin concentration of 50uM was the minimum that allowed detection of the free radical.

2.3.1.4 *Detection of doxorubicin free radical in viable intact MCF-7 cells.*

Doxorubicin free radical formation was investigated in a whole cell system. Doxorubicin [300uM] and MCF-7 cells [$3.5x10^6$] were incubated under anaerobic conditions at room temperature and measured by esr. Figure 2.5 shows the first derivative esr spectrum observed. The peak to peak width, 3.4 Gauss and g value, 2.00384 of this spectrum were typical of an organic free radical (reviewed by Foster, 1984). No signal was obtained in the absence of drug, MCF-7 cells or in the presence of air. Figure 2.6 shows the effect of time on the esr signal obtained in this system. The signal first appeared 25 minutes into the incubation and the intensity increased with time up to 85 minutes.

These results and those described in sections 2.3.1.1, 2.3.1.2, 2.3.1.3 and 2.3.1.4 indicated that doxorubicin stimulated NADPH oxidation,

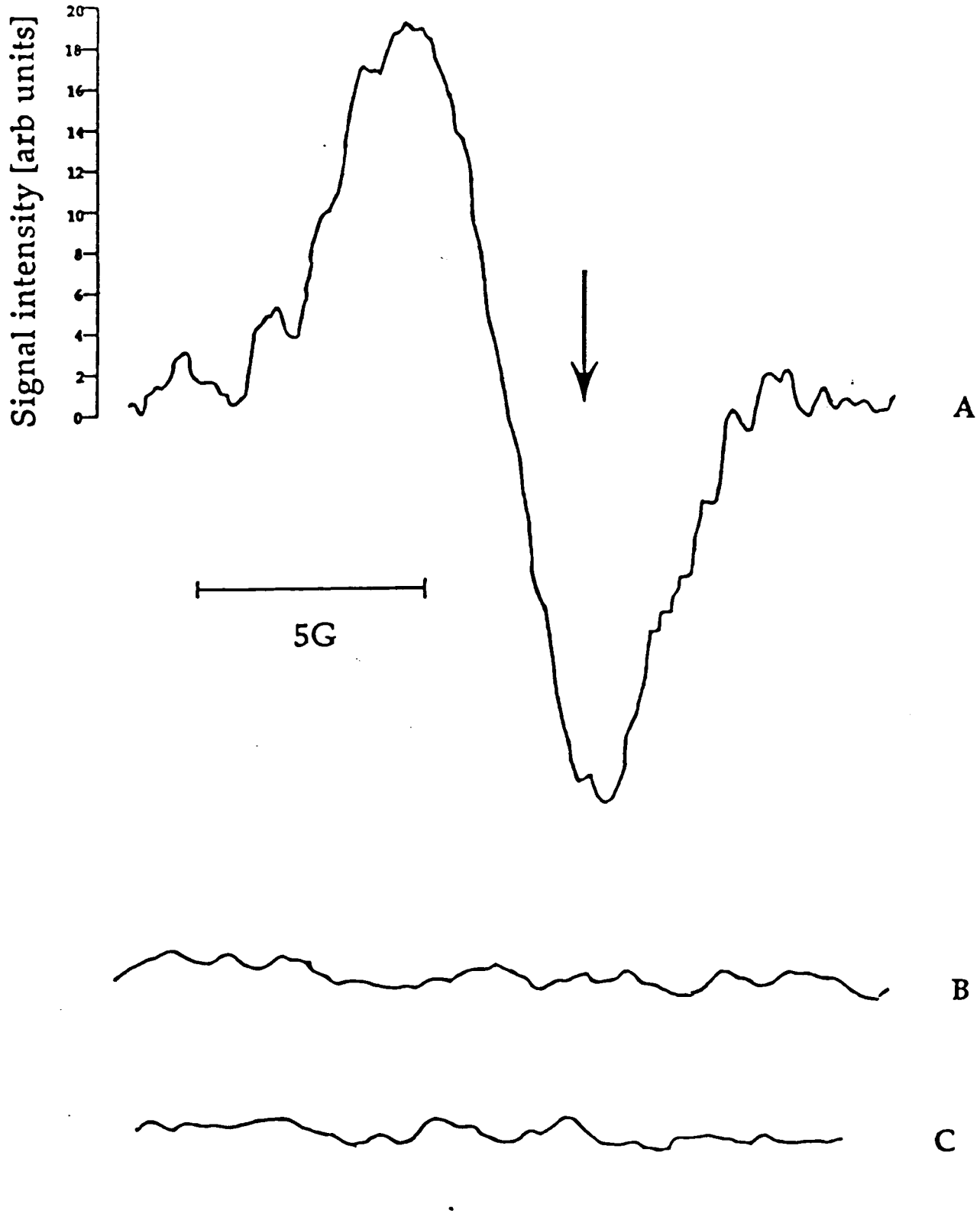


Figure 2.3 *Doxorubicin free radical in NADPH fortified MCF-7 S9 cell fraction detected by electron spin resonance spectrometry.*

Figure shows the first derivative esr spectrum obtained from an incubation of MCF-7 S9 fraction with NADPH [0.1mM] and doxorubicin [200uM], A: under anaerobic conditions, B: in the presence of oxygen and C: in the absence of drug.
Operating parameters were microwave frequency 9.465 GHz, microwave power 5mW, modulation amplitude 4G, time constant 1 second, scan time 4 minutes, receiver gain $2x10^5$ and scan range 40G. Arrow indicates DPPH g marker (2.0036G).

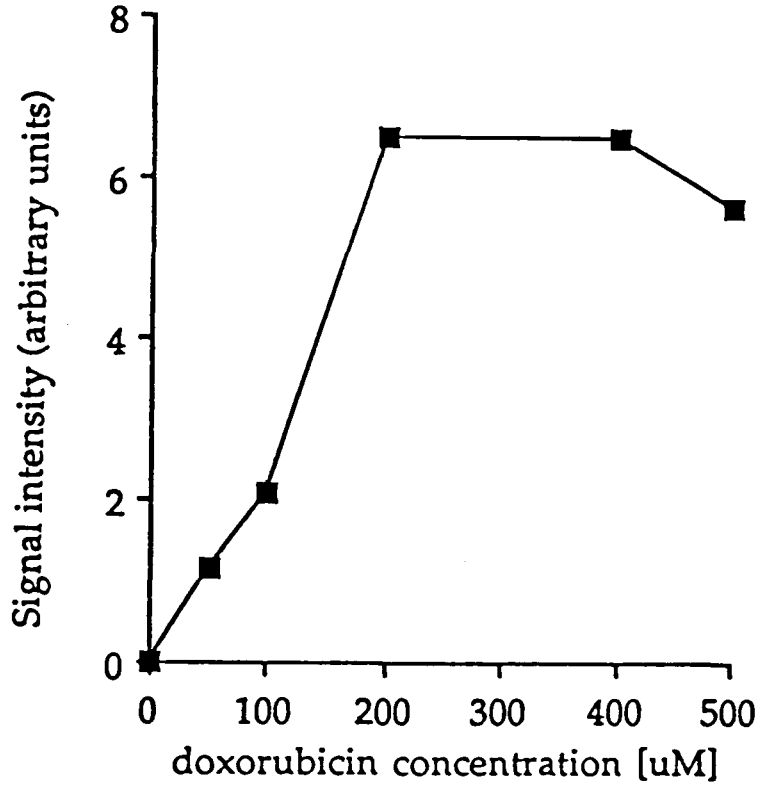


Figure 2.4 *Free radical formation by doxorubicin in NADPH fortified MCF-7 S9 cell fraction as determined by electron spin resonance spectrometry.*

Operating parameters were as described in figure 2.3

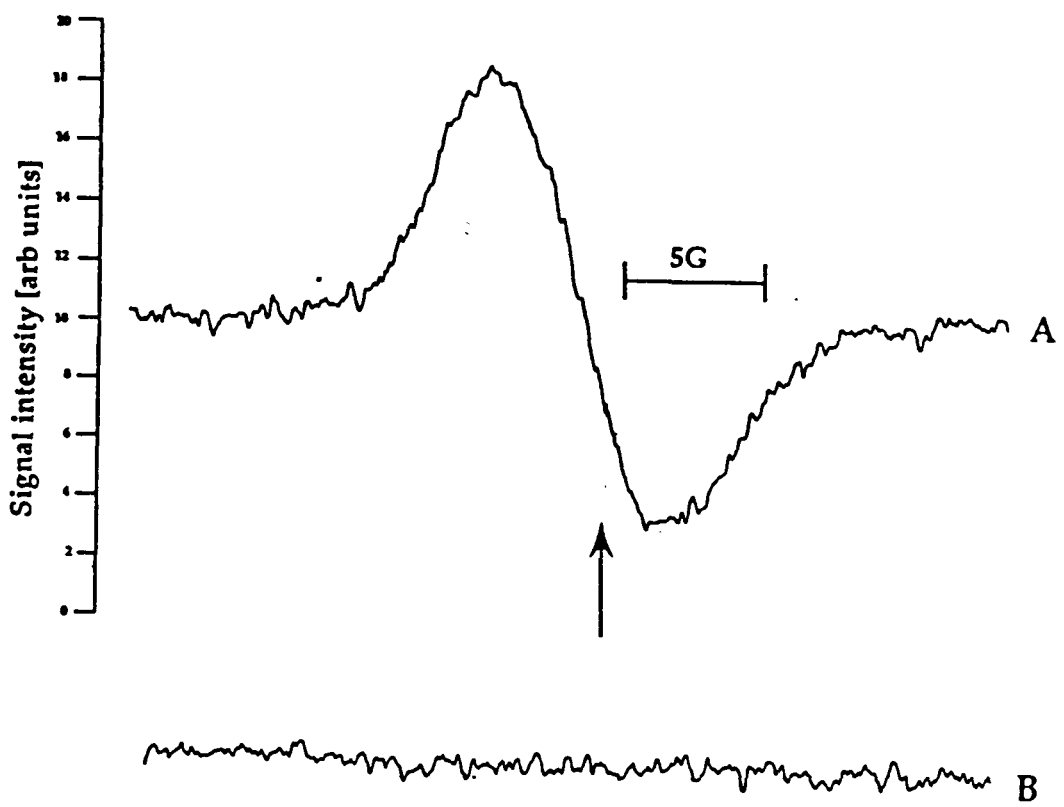


Figure 2.5 *Free radical formation by doxorubicin in MCF-7 cell suspension as determined by electron spin resonance spectrometry.*

Doxorubicin [300uM] was incubated with MCF-7 cells [$3.5x10^6 ml^{-1}$] in RPMI-1640 medium (serum supplemented) at room temperature under anaerobic conditions [A], under aerobic conditions [B] and in the absence of cells [C].

Operating parameters were microwave frequency 9.465 GHz, microwave power 5mW, modulation amplitude 4G, time constant 2 seconds, receiver gain $1.6x10^5$, scan time 16 minutes, and scan range 20G. Arrow indicates DPPH g marker (2.0036G).

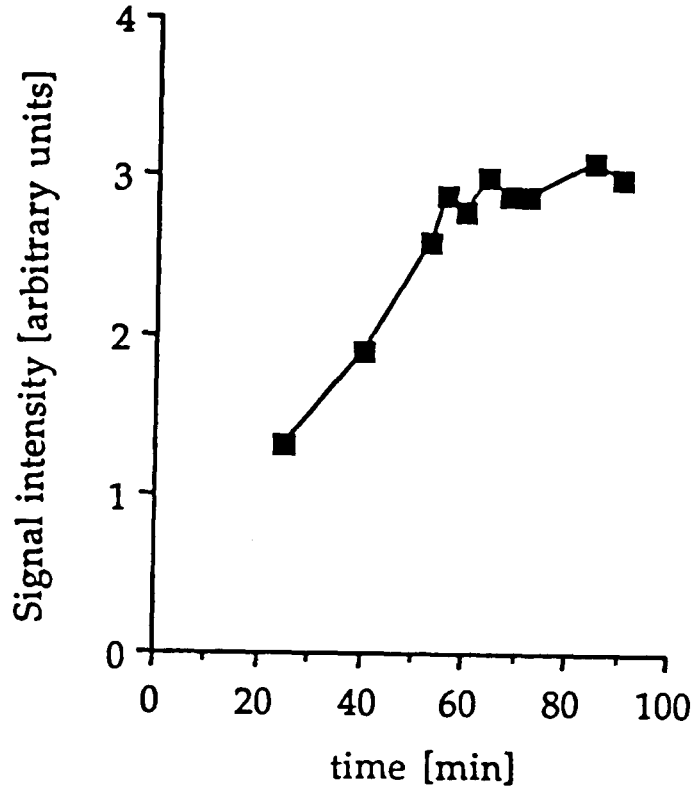


Figure 2.6 *Effect of time on free radical formed by doxorubicin in MCF-7 cells as determined by electron spin resonance spectrometry.*

For experimental conditions and operating parameters see figure 2.5.

superoxide anion formation and formed a free radical in MCF-7 S9 fraction and whole cells providing evidence for reductive activation of doxorubicin by MCF-7 cells. To further investigate the formation of oxygen radical species formed by doxorubicin in this system, spin trapping experiments were undertaken.

2.3.1.5 *Spin trapping of oxygen radicals formed by doxorubicin in MCF-7 S9 cell fraction*

The use of 5,5-dimethyl-1-pyrroline N-oxide [DMPO] as spin trap is detailed in section 1.12.2.

Figure 2.7A shows the first derivative esr spectrum obtained on incubation of doxorubicin [400uM] with NADPH fortified MCF-7 S9 fraction, desferrioxamine [1mM] and DMPO [100mM]. The hyperfine splitting constants for this spectrum which consisted of a 1:2:2:1 quartet were; A_N = 14.0G and A_H = 14.5G. The peak height ratios and splitting constants of this spectrum were consistant with the DMPO-hydroxyl radical spin adduct [DMPO-OH]. The calculated splitting constants for this adduct are $A_N = A_H$ = 14.87G [Britigan *et al.*, 1987].

To further ascertain whether this esr spectrum was due to doxorubicin dependent hydroxyl radical formation, the effect of catalase and dimethyl sulphoxide [DMSO] on the formation of the spectrum were investigated. In the absence of drug the signal intensity was reduced by 80% (figure 2.7B). In the presence of DMSO [16mM] and catalase [83 units] the signal intensity was reduced by 40% and 30% respectively [figure 2.7B]. Figure 2.8 shows the effect of DMSO and superoxide dismutase on the spin adduct generated by doxorubicin in MCF-7 S9 fraction. In the presence of SOD there was an increase [20%] in the peak height of the esr signal obtained. In the presence of DMSO (200mM) the 1:2:2:1 signal characteristic of the DMPO-OH adduct was replaced by a 1:1:1:1:1:1 sextet [see figure 2.8B] with hyperfine splitting constants of A_N= 16.25G and A_H= 23.0G. These values are consistent with

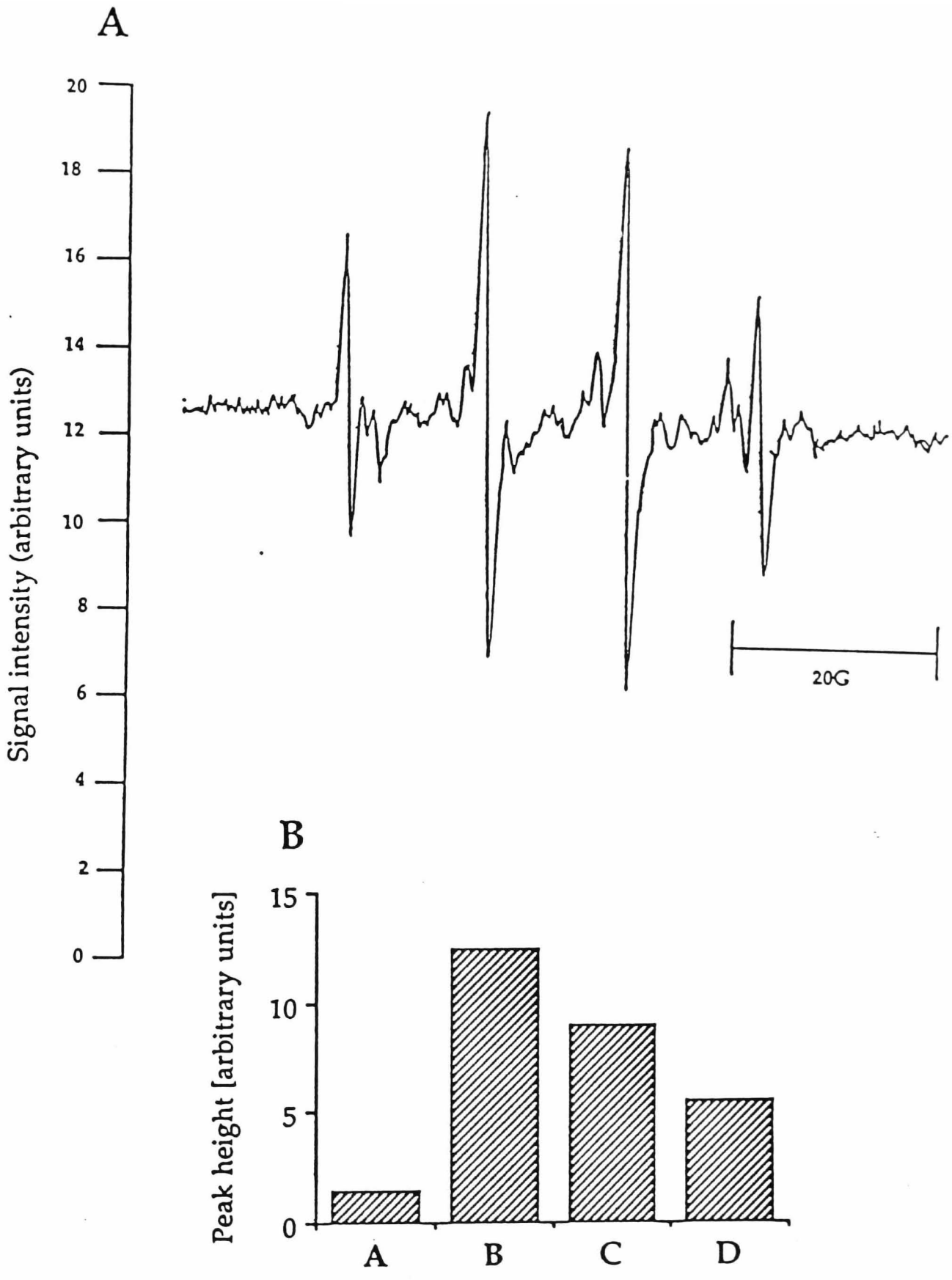
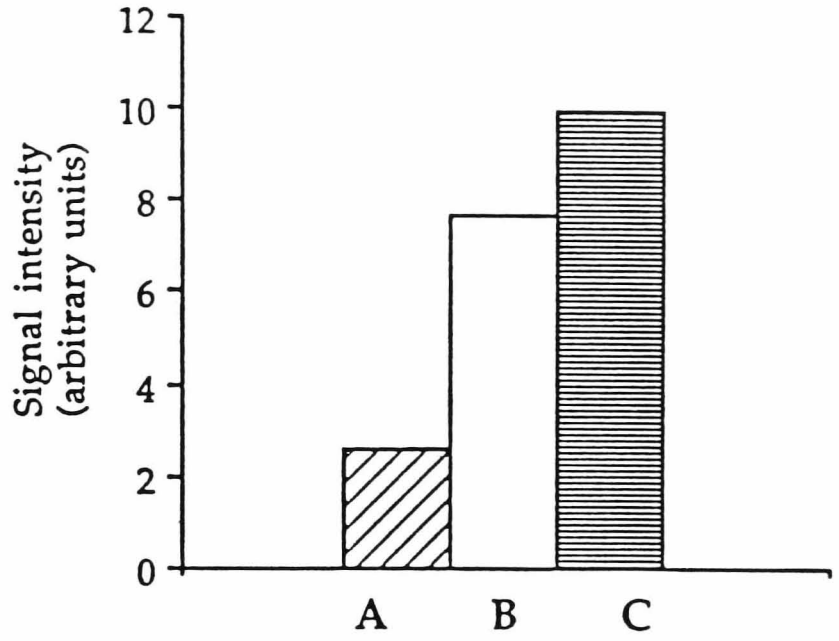


Figure 2.7 A: *Esr spectrum obtained from spin trapping studies of oxygen free radical formation by doxorubicin in NADPH fortified MCF-7 S9 cell fraction.*
The system consisted of doxorubicin [400uM], NADPH [0.1mM], desferrioxamine [1mM] and DMPO [100mM].
Operating parameters were microwave frequency 9.535 GHz, microwave power 10mW, modulation amplitude 0.5G, time constant 1 second, receiver gain 8.0×10^3, scan time 2 minutes and scan range 100G.

B: *The effect of oxygen free radical scavengers on the intensity of the esr signal obtained above.*
1: no drug, 2: doxorubicin [400uM], 3: doxorubicin [400uM] + catalase [83 units], 4: doxorubicin [400uM] + DMSO [16mM].

A



B

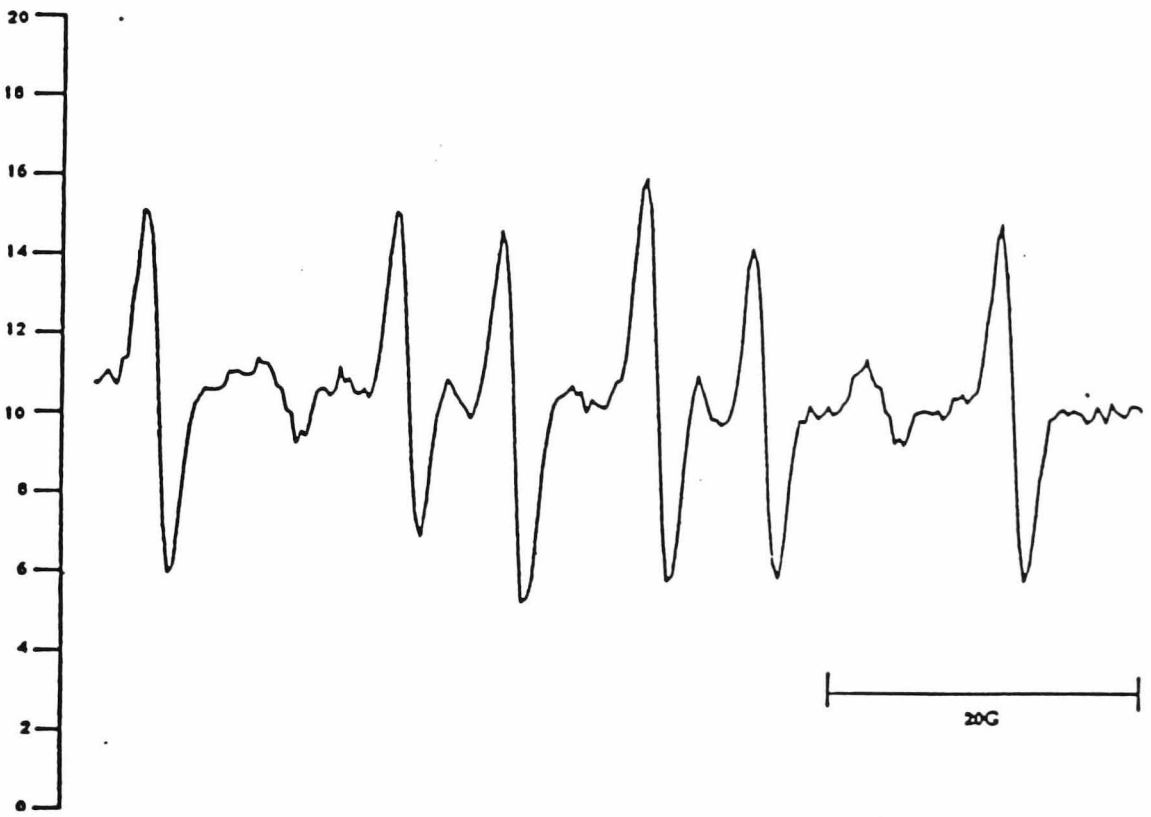


Figure 2.8 *Effect of oxygen free radical scavengers on the DMPO spin adduct formed on incubation of doxorubicin with NADPH fortified MCF-7 S9 cell fraction*

A. Intensities are derived from the spectra in figure 2.7. A: no drug, B: doxorubicin [400uM], C: doxorubicin + SOD [450 units].

B. The signal produced in the presence of DMSO (200mM). Esr parameters as described in figure 2.8A.

the spectra of the DMPO-methyl [DMPO-CH_3]] adduct which has calculated hyperfine splitting constants of A_N=15.31G and A_H= 23.0G [Pou *et al*,. 1989]. This adduct is formed after reaction of hydroxyl radical with DMSO to liberate a methyl radical which is then trapped by DMPO to give a DMPO-methyl radical spin adduct [see section 1.12.2].

2.3.1.6 *Spin trapping of oxygen radicals formed by doxorubicin in intact, viable MCF-7 cells.*

Section 2.1.4 showed that an organic free radical was detected in an incubation of doxorubicin with intact viable MCF-7 cells. In order to investigate oxygen free radical formation in this system spin trapping studies were carried out with freshly harvested MCF-7 cells. Figure 2.9A shows the first derivative esr spectrum observed from an incubation of MCF-7 cells [$2.5x10^5 ml^{-1}$] with doxorubicin [400uM] desferrioxamine [1mM] and DMPO [100mM]. The 1:2:2:1 quartet spectrum and hyperfine splitting constants [A_N = 14.0G, A_H = 14.5G] of this signal were characteristic of the DMPO-hydroxyl radical adduct [see section 2.3.1.5]. In the absence of drug a signal with reduced intensity (46%) was observed [figure 2.9B].

To further investigate the nature of the spectrum observed the effect of catalase was determined. Figure 2.9B shows that the presence of catalase [300 units] reduced the intensity of the spectrum produced by 41%.

In an attempt to discriminate whether the esr spectrum was associated with intra- or extracellular oxygen free radical formation the cell suspension was removed from the flat cell and the cells centrifuged [12,000g]. The supernatant was replaced in the flat cell and esr measurements continued. An identical spectrum was obtained [figure 2.9B] which was similar in intensity to that previously observed in the presence of cells.
To determine if oxygen was being utilised during the generation of free

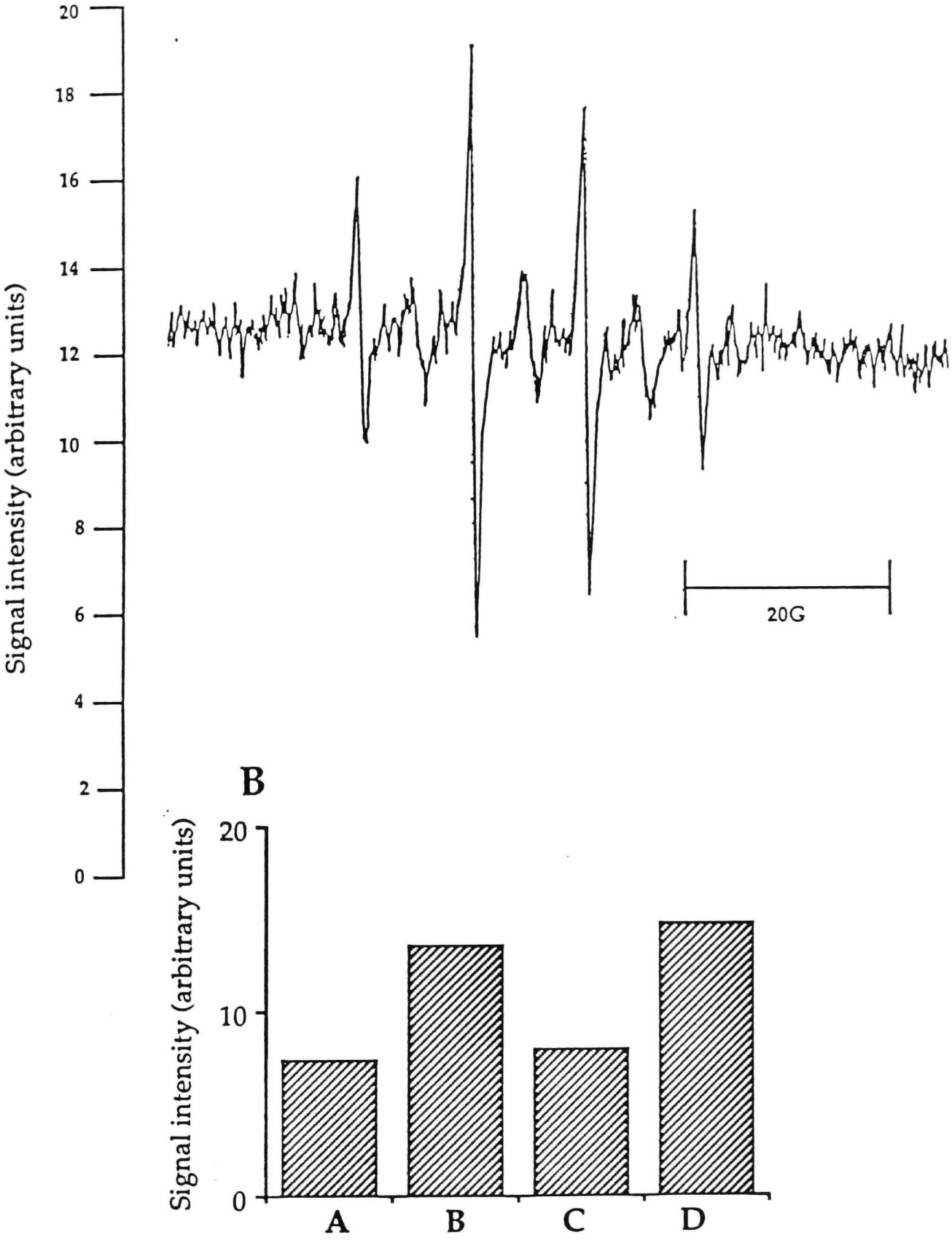


Figure 2.9 A: *Esr spectrum obtained from spin trapping studies of oxygen free radical formation by doxorubicin in intact viable MCF-7 cells.*

The incubation was carried out with freshly harvested MCF-7 cells [2.5 $10^5 ml^{-1}$] with doxorubicin [400uM], NADPH [0.5mM], desferrioxamine [0.1mM] and DMPO [100mM].

Esr operating parameters were as described in figure 2.7A.

B: *The effect of oxygen free radical scavengers on the signal intensity of the esr spectrum obtained above.*

A: No drug, B: doxorubicin [300uM], C: doxorubicin + catalase [300units] and D: MCF-7 cell supernatant.

radicals by doxorubicin in this system the effect of doxorubicin on oxygen consumption by MCF-7 cells was investigated.

2.3.1.7 *Effect of doxorubicin on oxygen consumption in intact, viable MCF-7 cells*

These studies were carried out as described in section 2.2.3. Figure 2.10 shows that doxorubicin stimulated oxygen consumption by MCF-7 cells in a concentration dependent manner.

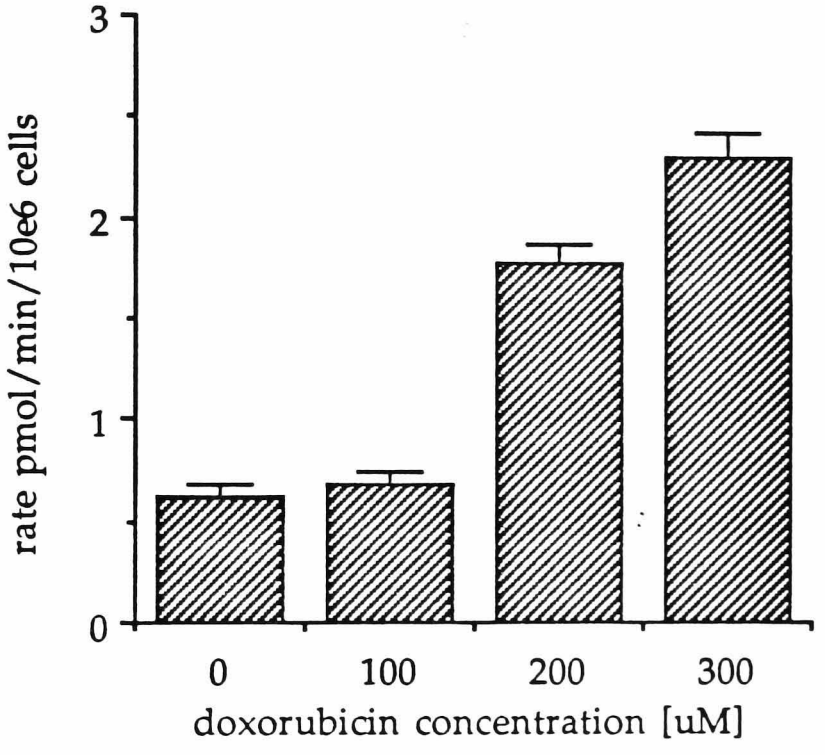


Figure 2.10 *Effect of doxorubicin on oxygen consumption by MCF-7 cells.*

Cell concentration was $10^6 ml^{-1}$. Data points are mean + standard deviation of three determinations.

2.3.2 *Investigation of free radical formation by mitozantrone and CI941 in MCF-7 cells*

These studies were carried out in MCF-7 S9 fraction. In most of the following studies a single drug concentration of 100uM was used. This represented a concentration at which stimulation of NADPH oxidation and superoxide formation were observed for doxorubicin [see sections 2.3.1.1 and 2.3.1.2].

2.3.2.1 *Effect of mitozantrone and CI941 on NADPH oxidation, superoxide anion generation, drug free radical formation and hydroxyl radical formation in MCF-7 S9 cell fraction.*

NADPH oxidation was determined as previously described [section 2.2.1]. Table 2.1 demonstrates that mitozantrone and CI941 [both 100uM] inhibited basal rate NADPH oxidation in MCF-7 S9 fraction by about 30%.

Table 2.2 shows the effect of mitozantrone and CI941 on superoxide generation in MCF-7 S9 fraction as determined by SOD inhibitable reduction of acetylated cytochrome c or adrenochrome formation [see sections 2.2.2 and 2.2.3]. These drugs produced a negligible amount of superoxide anion formation in this system. The effect of mitozantrone on reduction of acetylated cytochrome c in MCF-7 S9 fraction is shown in figure 2.11. No significant superoxide formation could be detected in the presence of mitozantrone.

Esr studies were carried out to determine if mitozantrone or CI941 formed a free radical species in NADPH fortified MCF-7 S9 fraction using identical experimental conditions as described for doxorubicin [see section 2.2.3]. Figure 2.12 shows that no esr signal could be detected for these drugs [300uM].

Table 2.1 *Effect of mitozantrone and CI941 on NADPH oxidation in MCF-7 S9 fraction.*

Drug [100uM]	NADPH oxidation [pmole $min^{-1}mg^{-1}$ S9 protein]
none	72 + 18
mitozantrone	49.7 + 2.2
CI941	48.96 + 2.9

Values are mean + sd of three determinations.

Table 2.2 *Effect Of mitozantrone and CI941 on superoxide anion formation in MCF-7 S9 fraction*

Drug [100μM]	Reduction of acetylated cytochrome c nmole $min^{-1}mg^{-1}$ S9 protein	adrenochrome formation $nmole^{1}min^{1}mg$ S9 protein
mitozantrone	0.98 + 2.09	6.93+0.51*
CI941	1.71 + 0.95*	4.42 + 1.9*

Values represent mean + sd of three determinations.
*(p,0.05)

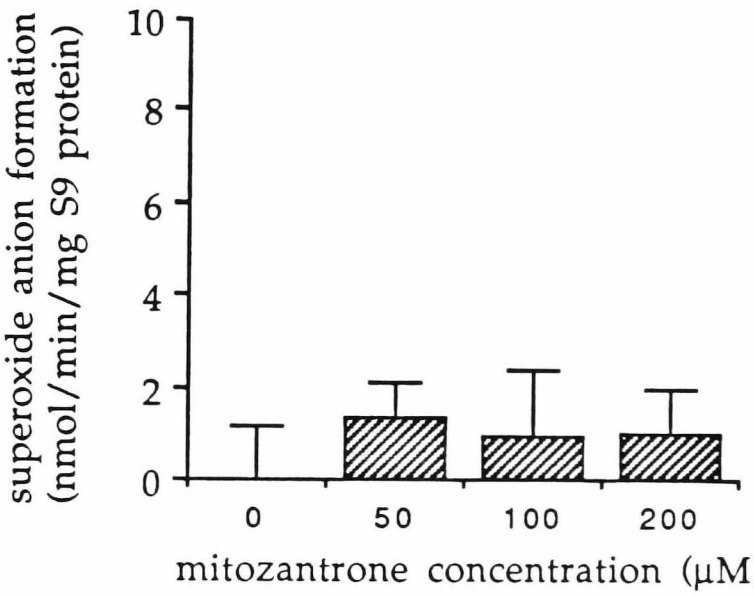


Figure 2.11 *Effect of mitozantrone on superoxide anion formation in MCF-7 S9 cell fraction as determined by reduction of acetylated cytochrome c.*

Data points are the mean + standard deviation of three determinations.

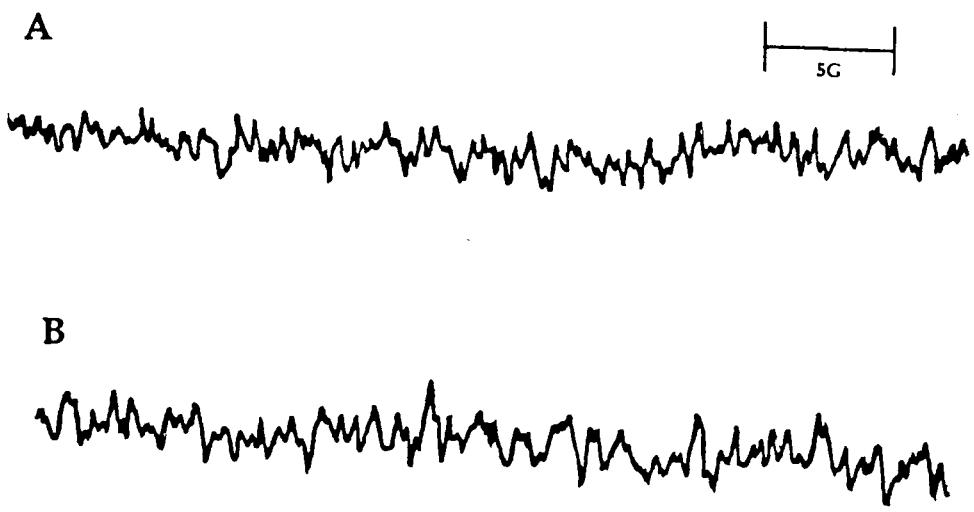


Figure 2.12 *Esr studies on free radical formation by mitozantrone and CI941 in NADPH fortified MCF-7 S9 cell fraction*

Spectra are:- **A**: mitozantrone [400uM] and **B**: CI941 [400uM].
Conditions and esr parameters were as described in figure 2.3.

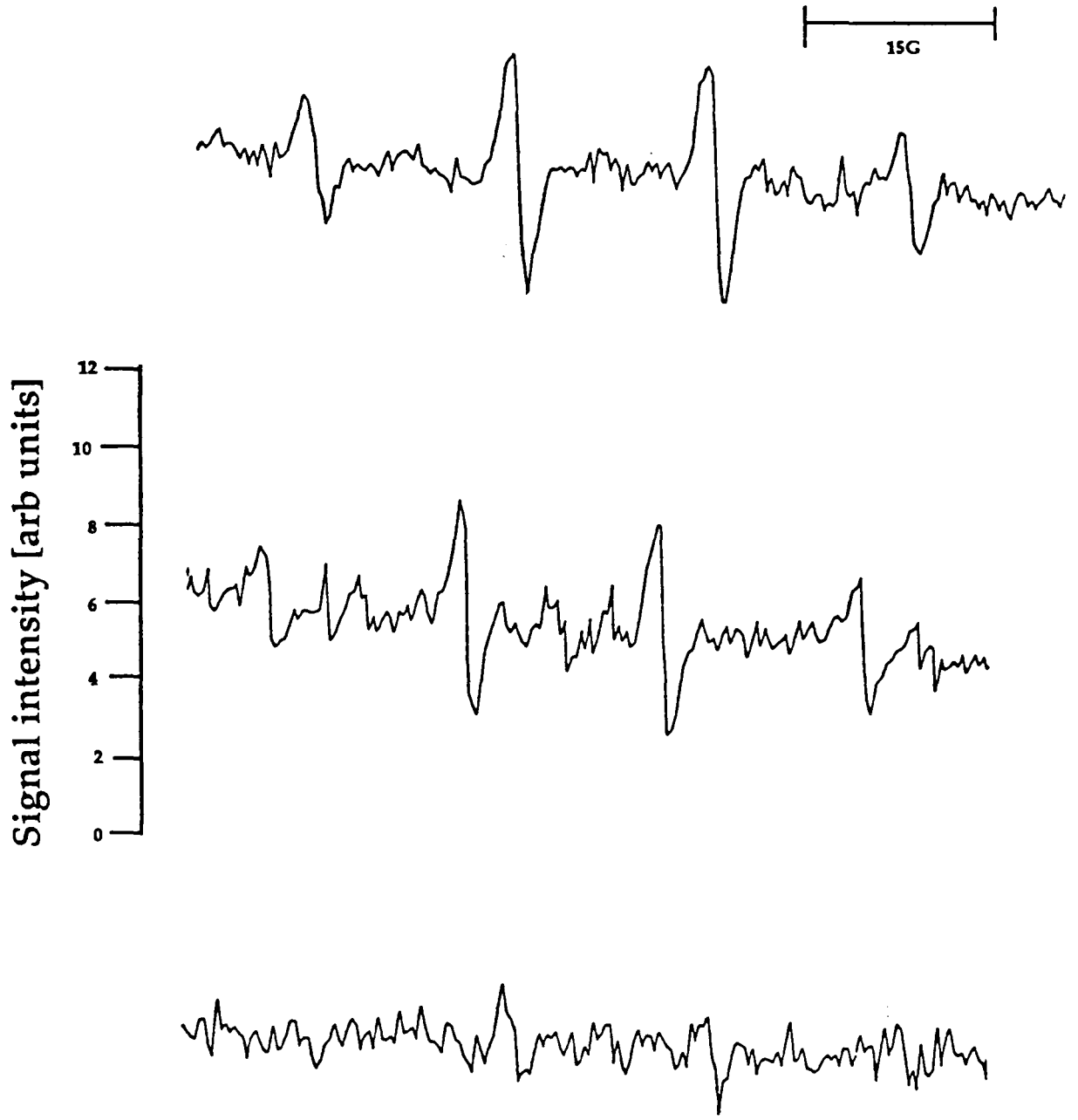


Figure 2.13 *Esr spectra from spin trapping studies of oxygen free radical formation by mitozantrone and CI941 in NADPH fortified MCF-7 S9 cell fraction.*

Incubations contained MCF-7 S9 fraction, NADPH [0.5mM], desferrioxamine [1mM] and DMPO [100mM] with **A**: no drug, **B**: mitozantrone [400uM] and **C**: CI941 [400uM]. Esr parameters were as described in figure 2.7.

Mitozantrone and CI941 mediated oxygen free radical formation in NADPH fortified MCF-7 cell S9 fraction was further investigated by esr using the spin trap DMPO [see section 2.2.5]. Figure 2.13 shows that no esr signal was observed in the presence of mitozantrone or CI941 in this system.

2.3.3 *Investigation of free radical formation by alkylaminoanthraquinones in MCF-7 cells*

2.3.3.1 *Effect of alkylaminoanthraquinones on NADPH oxidation in MCF-7 S9 cell fraction.*

Effect of a series of alkylaminoanthraquinones [AQ's] on NADPH oxidation in MCF-7 S9 fraction was determined as described in section 2.2.1. Figure 2.14 shows the effect of the AQ's on NADPH oxidation and demonstrates that 1AQ and 1,8AQ significantly stimulated base rate NADPH oxidation, whilst 1,4AQ and 1,5AQ produced no significant effect.

Since 1AQ produced the largest stimulation of base rate NADPH oxidation [440%], this compound was investigated further. Figure 2.15 shows that 1AQ stimulated MCF-7 S9 base rate NADPH oxidation in a concentration dependent manner.

2.3.3.2 *Effect of alkylaminoanthraquinones on superoxide anion formation in MCF-7 S9 cell fraction.*

In order to substantiate further whether the AQ's could undergo reductive activation in MCF-7 S9 fraction, superoxide anion formation by these compounds was investigated by the methods described previously [section 2.2.2 and 2.2.3].

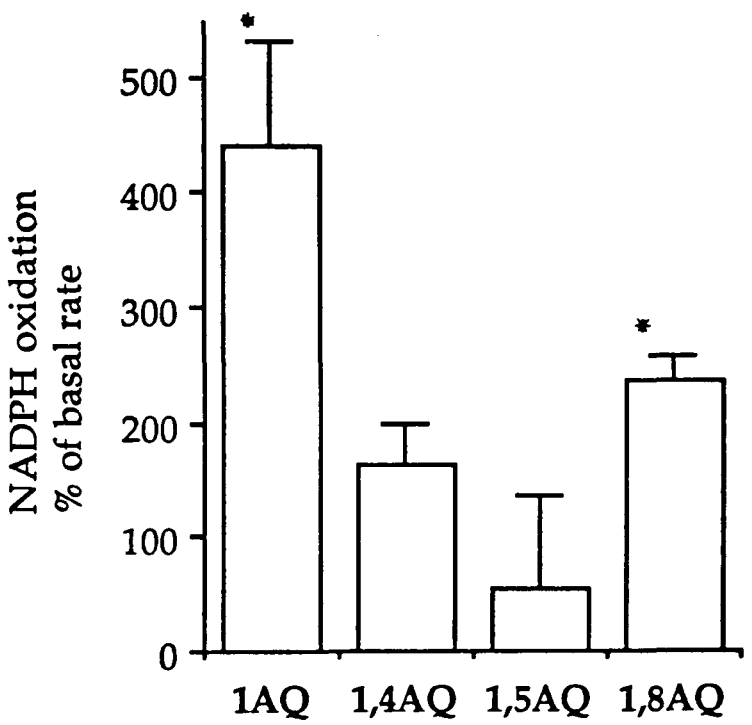


Figure 2.14 *Effect of alkylaminoanthraquinones on NADPH oxidation in MCF-7 S9 cell fraction.*

AQ concentration was 100uM. Base rate NADPH oxidation was 72 + 18 pmole $min^{-1}mg^{-1}$ S9 protein. Data points are the mean + standard deviation of three determinations.* $p<0.01$

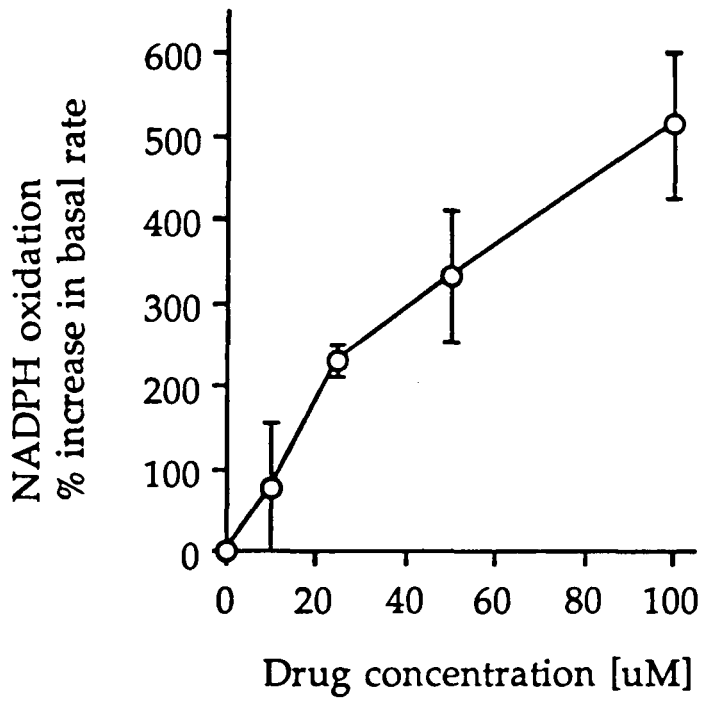


Figure 2.15 *Effect of 1AQ on NADPH oxidation in MCF-7 S9 cell fraction.*

Base rate NADPH oxidation was 81 pmole $min^{-1}mg^{-1}$S9 protein.
Data points are the mean + standard deviation of three determinations.

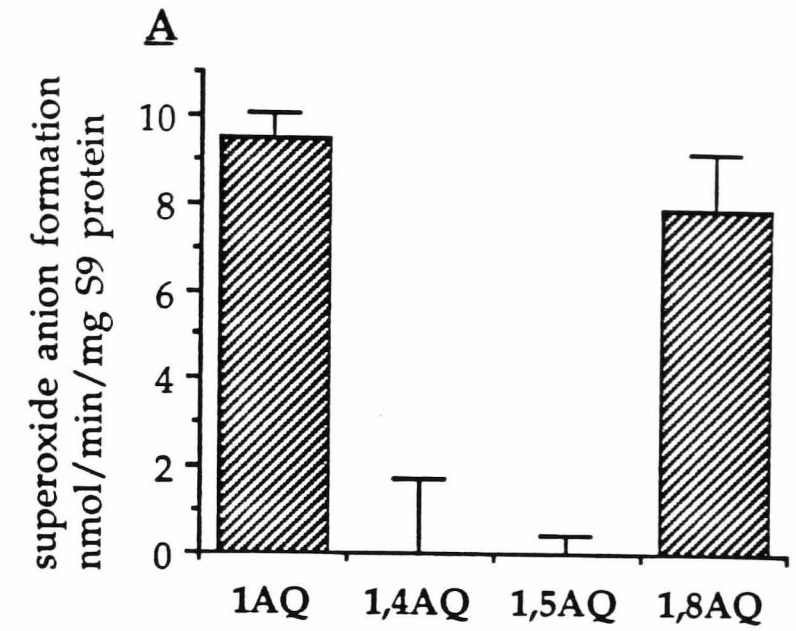
1AQ and 1,8AQ both stimulate superoxide anion formation in MCF-7 S9 fraction as determined by reduction of acetylated cytochrome c, however 1,4AQ and 1,5AQ produced no significant effect (figure 2.16A). 1AQ stimulated superoxide anion formation in a concentration dependent manner in this system (figure 2.17). AQ mediated superoxide formation was also investigated using the adrenochrome assay. Figure 2.15B shows that using this assay 1AQ, 1,4AQ and 1,8AQ were observed to significantly stimulate superoxide anion formation in MCF-7 S9 fraction, whilst 1,5AQ produced no significant effect.

2.3.3.3 *Detection of alkylaminoanthraquinone free radical formation in NADPH fortified MCF-7 S9 fraction*

Esr studies were carried out under anaerobic conditions to determine whether stimulation of NADPH oxidation and superoxide anion formation by AQ's was associated with semiquinone formation. Experimental conditions as previously described for doxorubicin were used [section 2.2.3]. Figure 2.18 shows the first derivative esr spectra obtained for AQ's in NADPH fortified MCF-7 S9 fraction. It can be seen from this figure that esr spectra were detected for 1,5AQ and 1,8AQ whereas no spectra were obtained for 1AQ or 1,4 AQ. The spectrum observed for 1,5AQ had a peak to peak width of 4.85G and a g value of 2.00455. whilst the spectrum observed for 1,8AQ had a peak to peak width of 4.2G and a g value of 2.00461. The parameters of these spectra are characteristic of organic free radicals [reviewed by Foster, 1984)]. The esr spectra obtained from these compounds were dependent on the absence air.

2.3.3.4 *Spin trapping of oxygen free radicals formed by alkylaminoanthraquinones in MCF-7 S9 cell fraction*

Studies were carried out in NADPH fortified MCF-7 S9 fraction as described in section 2.2.5. Figures 2.19, 2.20 and 2.21 show that spin adducts could be detected for all four AQ's in this system. The peak size ratios and hyperfine splitting constants for the spectra obtained



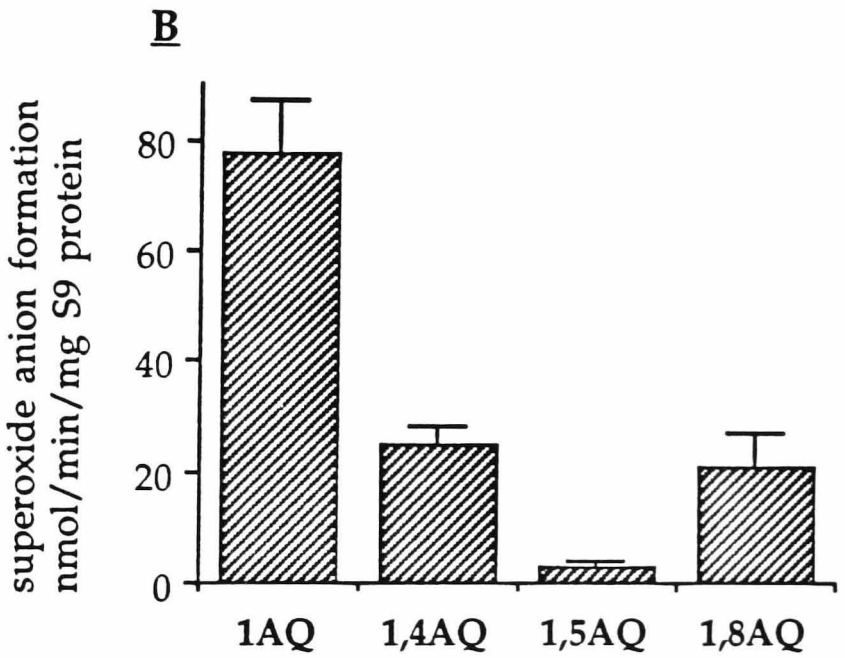


Figure 2.16 *Effect of alkylaminoanthraquinones on superoxide anion formation in MCF-7 S9 cell fraction as determined by reduction of acetylated cytochrome c [A] and adrenochrome formation [B].*

AQ concentration was 100uM.
Data points are the mean + standard deviation of three determinations.

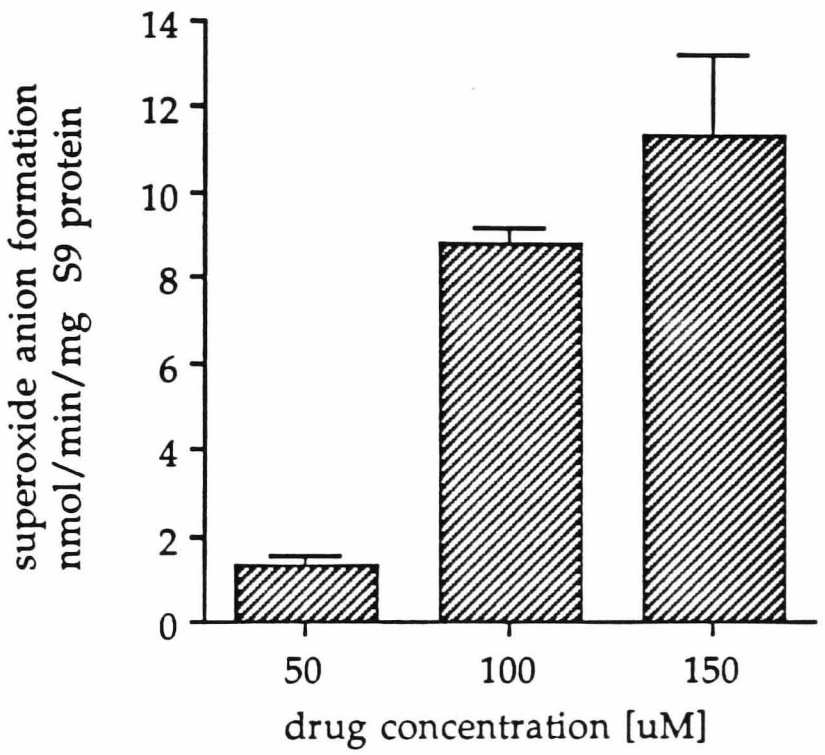


Figure 2.17 *Effect of 1AQ on superoxide anion formation in MCF-7 S9 cell fraction as determined by reduction of acetylated cytochrome c.*

Data points are the mean + standard deviation of three determinations.

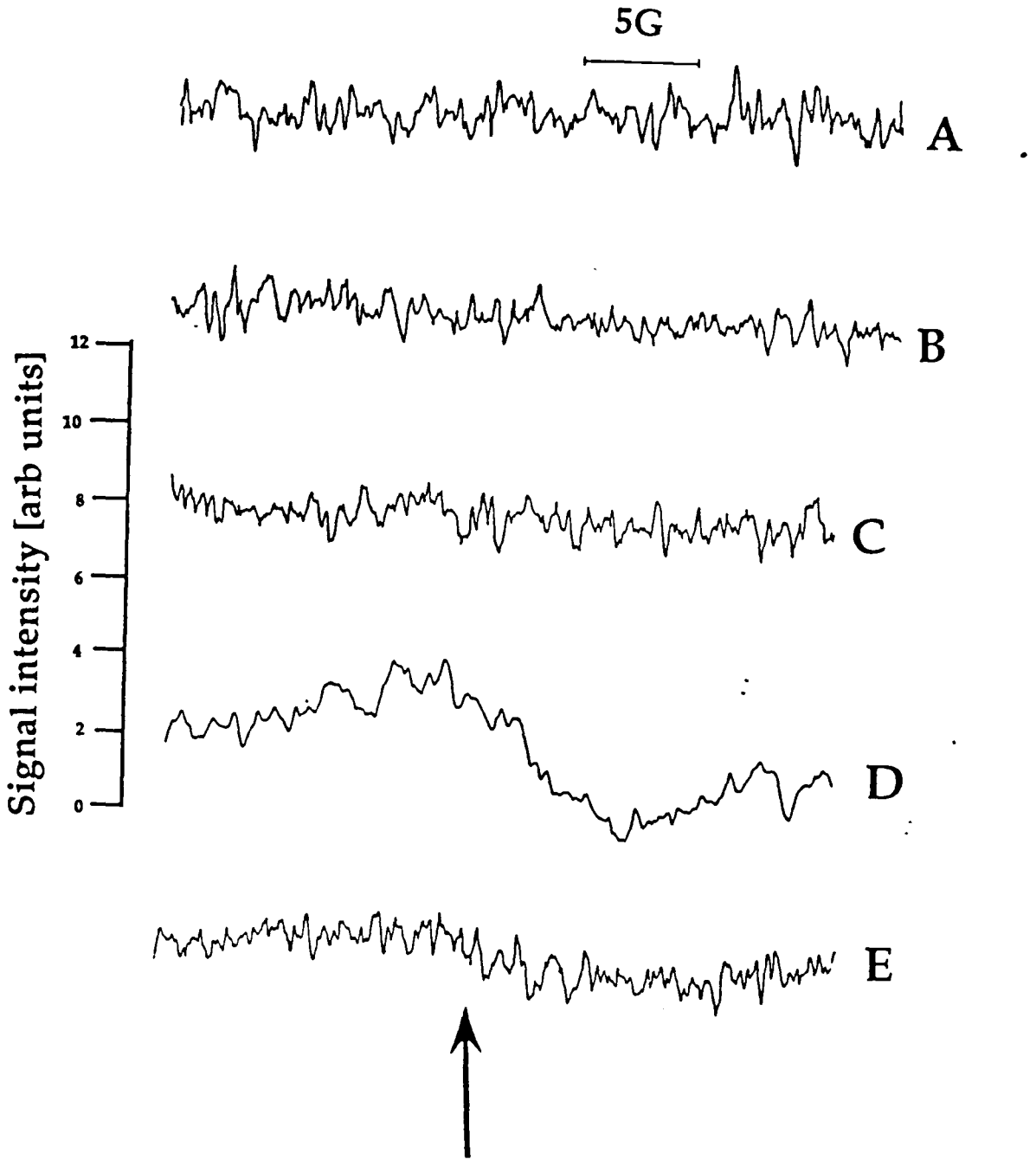


Figure 2.18 *Free radical formation by alkylaminoanthraquinones in NADPH fortified MCF-7 S9 cell fraction as determined by electron spin resonance spectrometry.*

No Drug **[A]**, 1AQ **[B]**, 1,4AQ **[C]**, 1,5AQ **[D]**, or 1,8AQ **[E]** (300uM) were incubated with NADPH fortified MCF-7 S9 cell fraction under anaerobic conditions at room temperature.

The operating parameters were: microwave frequency 9.455 GHz, microwave power 5mW, modulation amplitude 4G, time constant 2 seconds, scan time 16 minutes, scan range 20G and receiver gain 2.5×10^5 [except for 1,8AQ (3.2×10^5)]. Arrow indicates DPPH g-marker [2.0036].

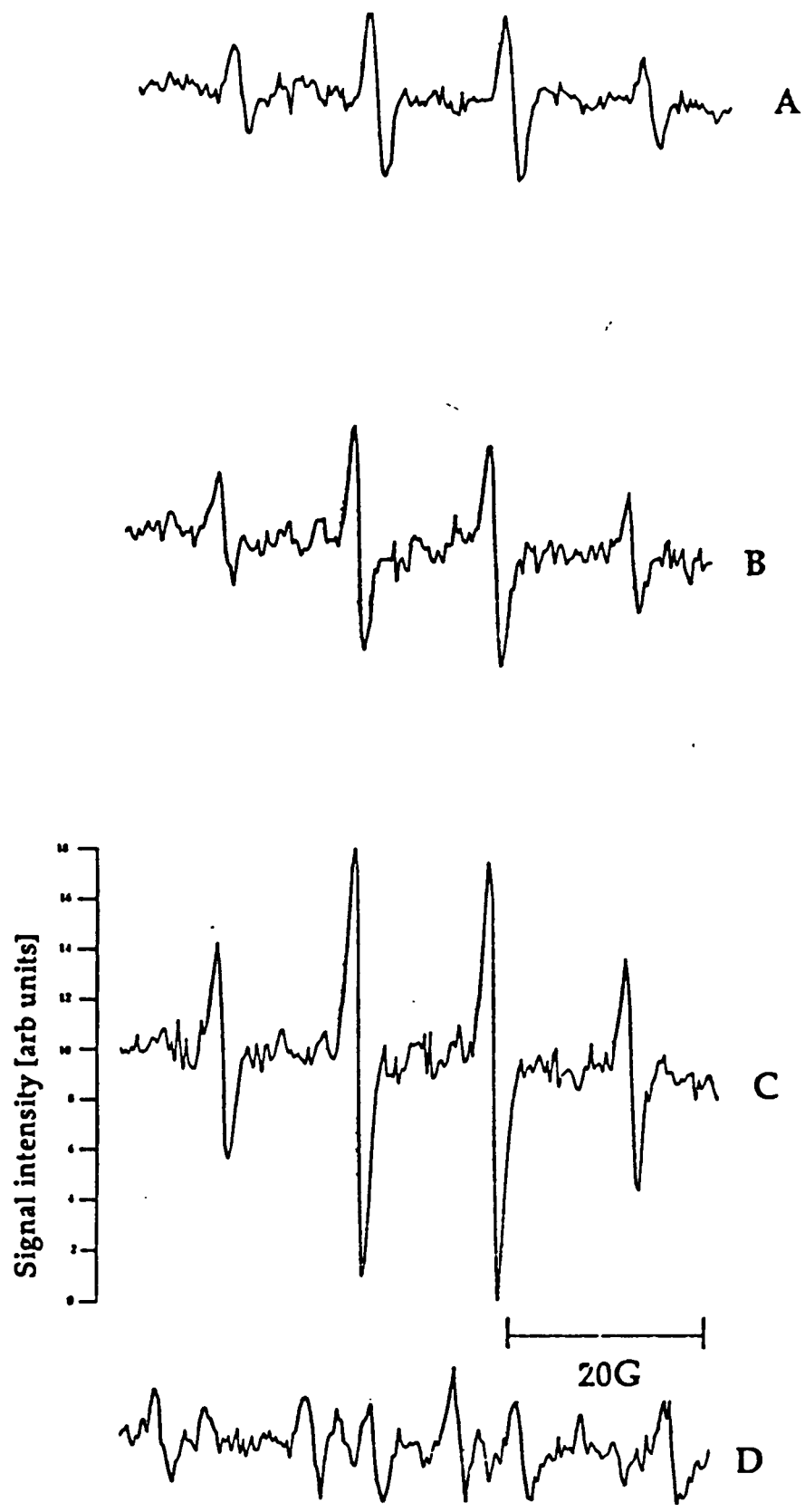


Figure 2.19 *Esr spectra from spin trapping studies of oxygen free radical formation by 1AQ in NADPH fortified MCF-7 S9 cell fraction*

The system consisted of S9 fraction, NADPH [0.5mM], desferrioxamine [1mM] and DMPO [100mM] with: A: no drug, B: 1AQ [400uM](-S9 fraction), C: 1AQ [400uM], D: 1AQ + DMSO [200mM].
Esr parameters were as described in figure 2.7.

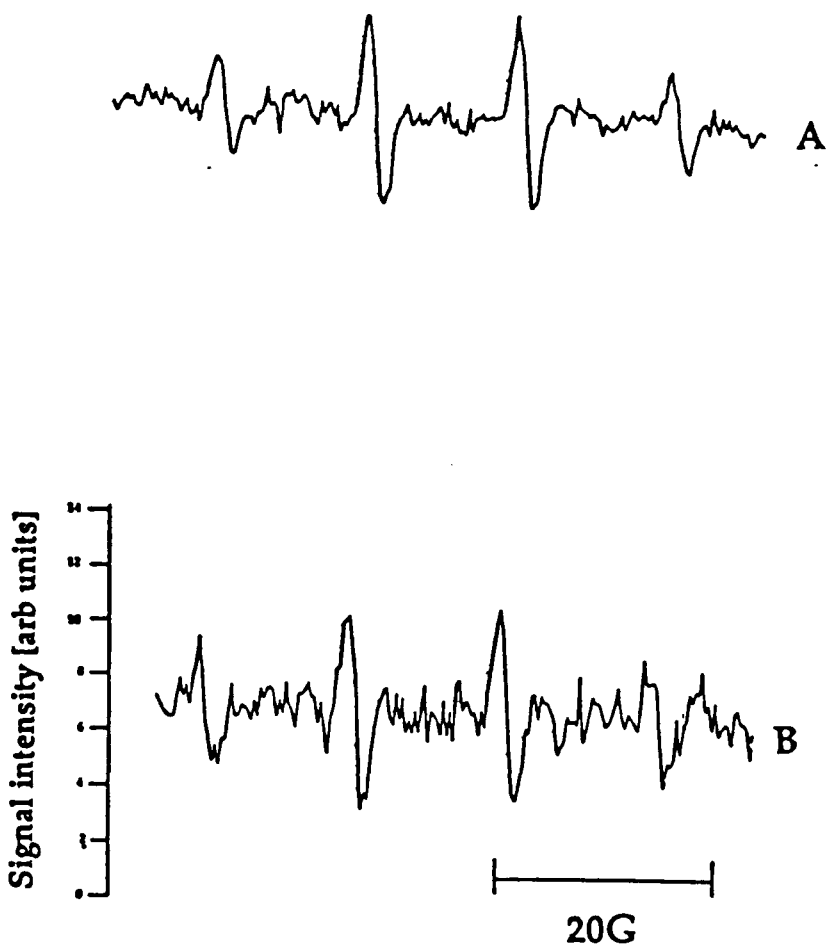


Figure 2.20 *Esr spectra from spin trapping studies of oxygen free radical formation by 1,4AQ in NADPH fortified MCF-7 S9 cell fraction*

The system consisted of S9 fraction, NADPH [0.5mM], desferrioxamine [1mM] and DMPO [100mM] with: **A**: no drug, **B**: 1,4AQ [400uM], **C**: 1,4AQ [400uM]-S9 fraction. Esr parameters were as described in figure 2.7.

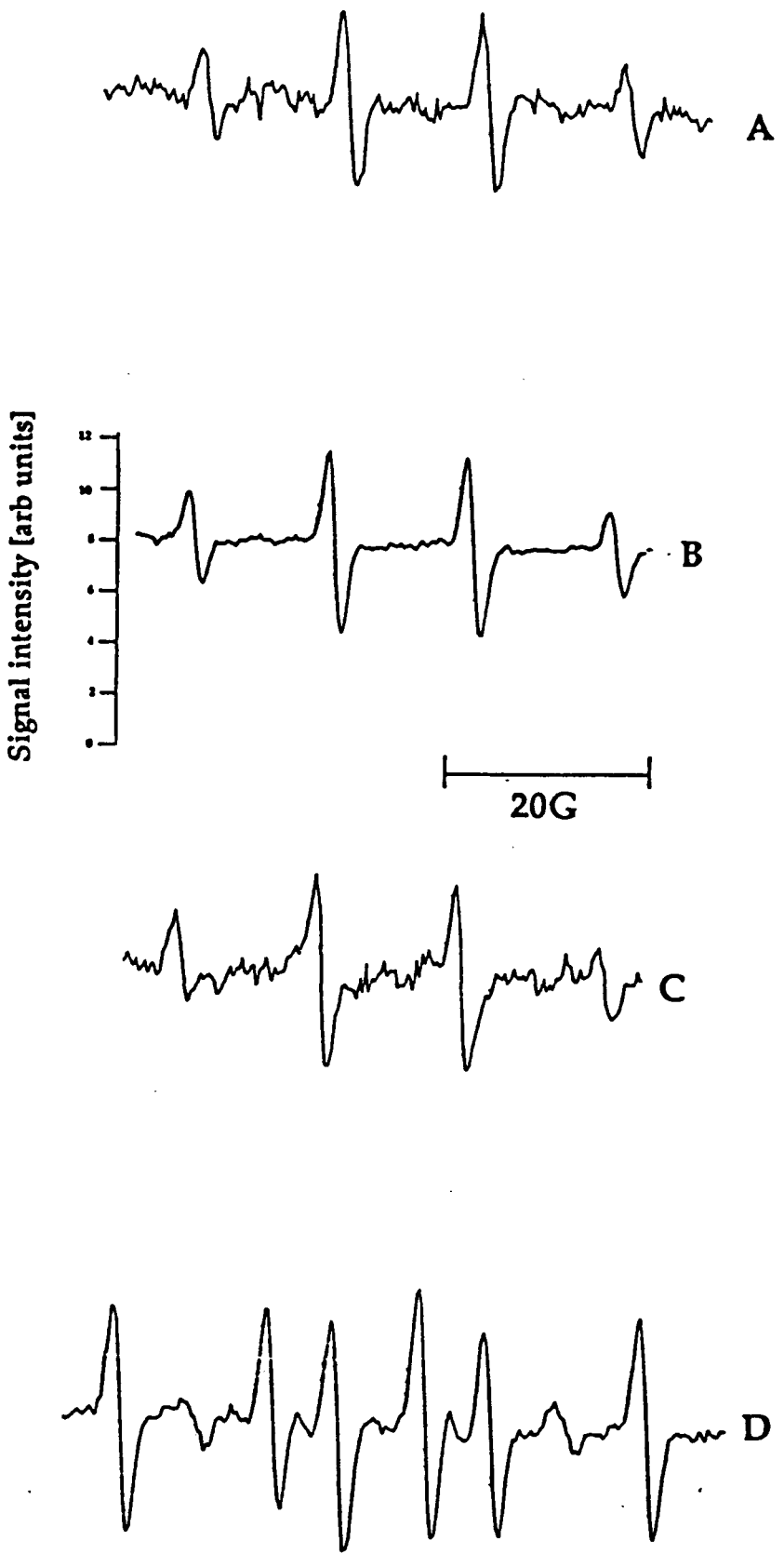


Figure 2.21 *Esr spectra from spin trapping studies of oxygen free radical formation by 1,5AQ in NADPH fortified MCF-7 S9 cell fraction*

The system consisted of S9 fraction, NADPH [0.5mM], desferrioxamine [1mM] and DMPO [100mM] with: A: no drug, B: 1,5AQ [400uM]-S9 fraction, C: 1,5AQ [400uM] D: 1,5AQ + DMSO [200mM].
Esr parameters were as described in figure 2.7 (except C where gain = 4.0×10^3).

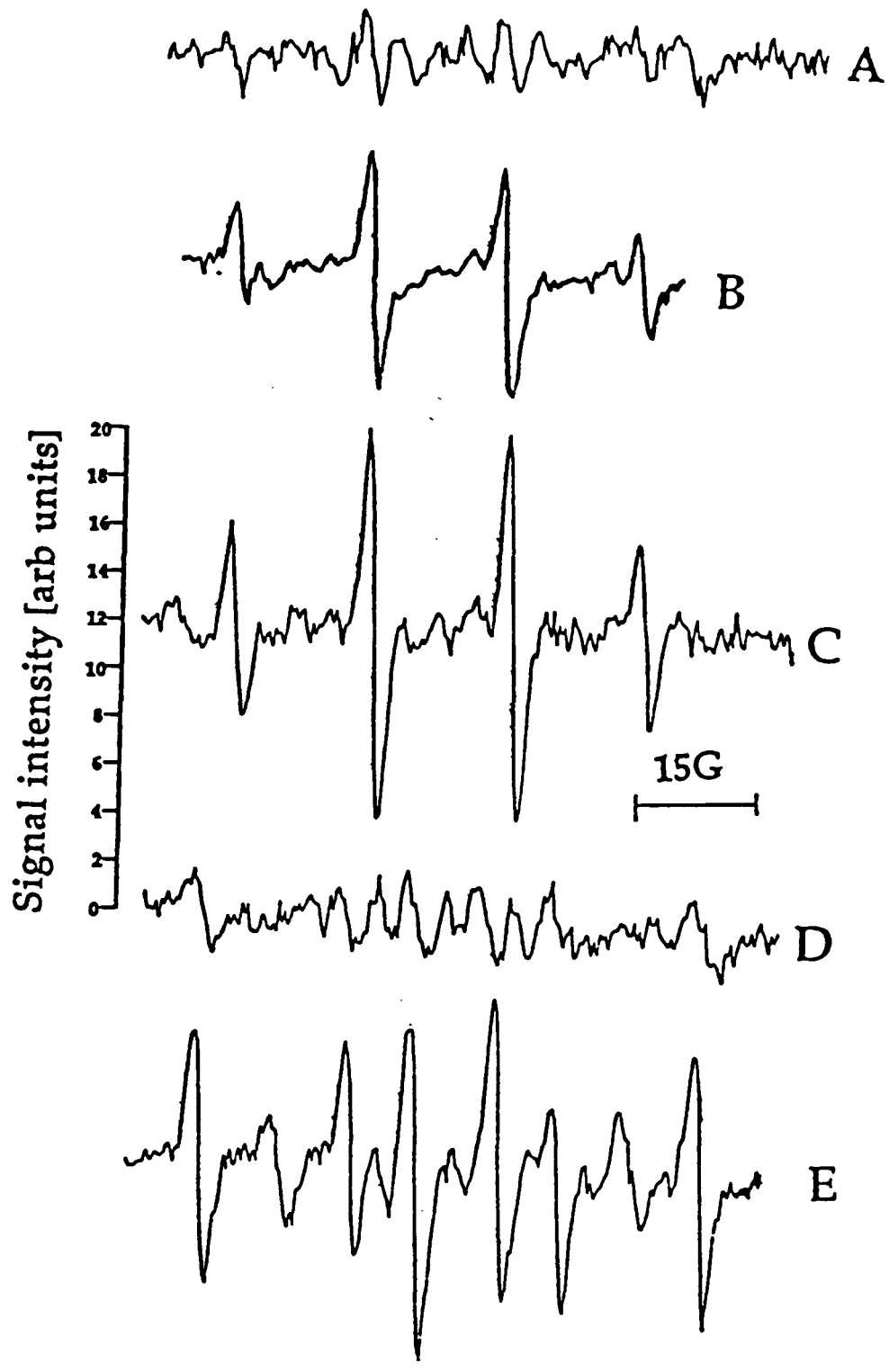


Figure 2.22 *Esr spectra from spin trapping studies of oxygen free radical formation by 1,8AQ in NADPH fortified MCF-7 S9 cell fraction*

The system consisted of S9 fraction, NADPH [0.5mM], desferrioxamine [1mM] and DMPO [100mM] with: **A**: no drug, **B**: 1,8AQ [400uM], **C**: 1,8AQ + S9 fraction, **D**: 1,8AQ + catalase [300 units ml^{-1}] and **E**: 1,8AQ + DMSO [200mM]. Esr parameters were as described in figure 2.7.

given in table 2.3 are consistent with the DMPO-OH adduct [see section 2.3.1.5]. In the absence of S9 fraction 1AQ, 1,5AQ and 1,8AQ produced similar but less intense esr signals. However, a signal of greater intensity was detected for 1,4AQ in the absence of S9 fraction. The net intensities of the spectra obtained attributable to the presence of S9 are shown in figure 2.23.

To further investigate whether the spectra obtained by the AQ's in MCF-7 S9 fraction were due to drug mediated hydroxyl radical formation, the incubations were repeated in the presence of DMSO [200mM]. Table 2.3 shows the hyperfine splitting constants for the esr spectra obtained. In each case a 1:1:1:1:1:1 signal was produced with hyperfine splitting constants consistent with the DMPO-CH_3 adduct (Pou *et al.*, 1989) [see figures 2.19, 2.20, 2.21 and 2.22].

The nature of the spectra obtained in the presence of 1AQ and 1,8AQ were investigated in more detail. Figure 2.24 shows that catalase [83 units] and DMSO [16mM] reduced the intensity of the spectrum obtained for 1AQ . Figure 2.22 shows the spectrum obtained for 1,8AQ in the presence of catalase [300 units ml^{-1}]. Catalase abolished the drug dependent esr signal, as shown in figure 2.22. The relative intensities of the spectra obtained for 1,8AQ in MCF-7 S9 fraction in the presence of DMSO or catalase are shown in figure 2.25.

Table 2.3 *Spin adducts obtained from spin trapping experiments with alkylaminoanthraquinones in NADPH fortified MCF-7 S9 fraction.*

alkylamino-anthraquinone	*Hyperfine splitting constants* -DMSO A_N	A_H	+DMSO A_N	A_H	*Signal description* -DMSO	+DMSO
1AQ	15.0	15.0	16.25	22.75	1:2:2:1	1:1:1:1:1:1
1,4AQ	14.75	15.0	16.25	23.0	"	"
1,5AQ	15.0	15.0	16.25	23.0	"	"
1,8AQ	15.0	15.0	16.25	23.0	"	"

Drug concentration was 400uM. DMSO concentration was 200mM.
Esr parameters were as described in section 2.2.5.

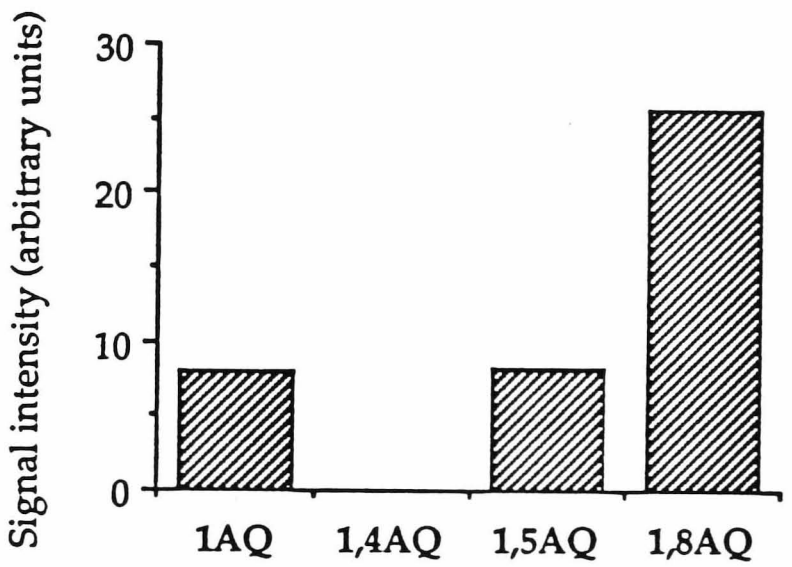


Figure 2.23 *Signal intensities of esr spectra obtained from spin trapping studies of oxygen free radical formation by alkylaminoanthraquinones in NADPH fortified MCF-7 S9 cell fraction*

Incubations were carried out in MCF-7 S9 fraction in the presence of NADPH [0.5mM], desferrioxamine [1mM], DMPO [100mM] and drug [400uM]. Esr parameters were as described in figure 2.7.

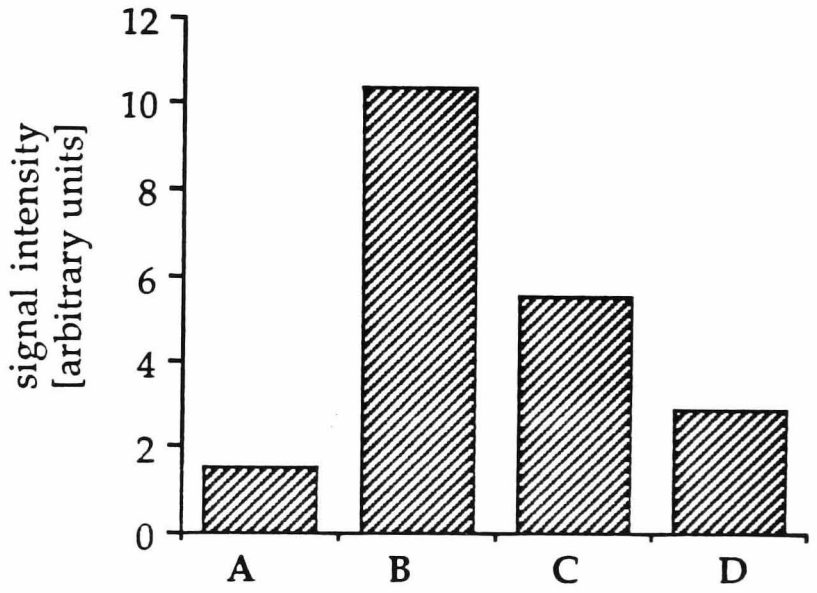


Figure 2.24 *Signal intensities of the esr spectra obtained from spin trapping studies of oxygen free radical formation by 1AQ in NADPH fortified MCF-7 S9 cell fraction.*

Figure 2.19 shows the spectra obtained.
A: no drug, **B**: 1AQ [400uM], **C**: 1AQ + catalase [83 units] and **D**: 1AQ + DMSO [16mM].

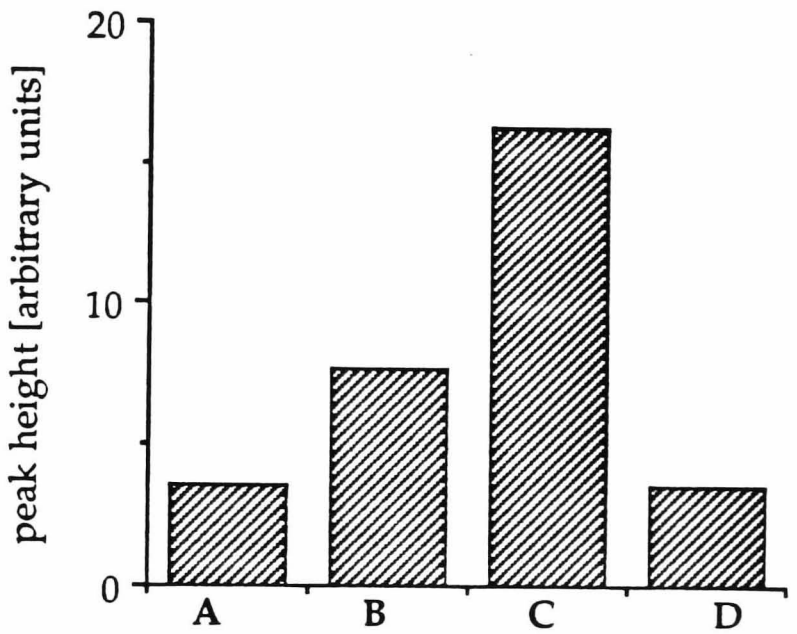


Figure 2.25 *Signal intensities of esr spectra obtained from spin trapping studies of oxygen free radical formation by 1,8AQ in NADPH fortified MCF-7 S9 cell fraction*

Intensities were derived from figure 2.22.
A: no drug, **B**: 1,8AQ [400uM] minus S9 fraction **C**: drug [400uM] **D**: drug + catalase [300 units ml^{-1}].

2.4 Discussion

2.4.1 *Redox cycling by doxorubicin in MCF-7 cells*

Studies were carried out in order to determine if MCF-7 cells were capable of reductively activating doxorubicin to a free radical species by a redox cycling mechanism [see section 2.3.1]. This has widely been reported to occur in animal and human liver and heart tissues [see section 1.16.4.1] but no comprehensive studies have been carried out in human tumour tissue. As discussed in section 1.16.5 redox cycling has been implicated in the cardiotoxicity of doxorubicin, more so than in the antitumour activity. MCF-7 human breast cancer cells, originally isolated from a female patient with metastatic breast cancer (Seoule *et al.*, 1973), is a particularly relevant cell line for these investigations as doxorubicin (and mitozantrone) are used in the chemotherapy of advanced breast cancer (see sections 1.13 and 1.14).

Doxorubicin was found to stimulate in a concentration dependent manner both NADPH oxidation and superoxide anion formation in MCF-7 S9 cell fraction [see figures 2.1 and 2.2]. This indicated that doxorubicin was being metabolised by NADPH requiring enzymes with concomitant reduction of molecular oxygen. Furthermore, these events were associated with formation of an organic free radical species observed by esr [figure 2.3]. The dependence of this esr signal on the presence of doxorubicin, NADPH-fortified MCF-7 S9 cell fraction and absence of oxygen indicated that this was a metabolically generated doxorubicin semiquinone free radical. Moreover, the intensity of this esr signal was dependent on the concentration of doxorubicin. The doxorubicin semiquinone free radical is the one electron reduction product of doxorubicin following reduction by NADPH requiring flavin reductase enzymes. The esr signal has a g value similar to that of the chemically generated doxorubicin semiquinone (Lown and Chen, 1981). The broadline esr signal produced [figure 2.3] has been proposed to be due to hindered rotation of the doxorubicin free radical as a consequence of membrane binding (Kalyanaraman *et al.*, 1980). The esr signal observed did not appear

until thirty minutes into incubation, this is likely to be due to residual oxygen in the incubate which needs to be reduced [see figure 1.19] during metabolic activation of doxorubicin before a steady state esr signal for the semiquinone free radical can be detected.

The above findings are consistent with doxorubicin undergoing one electron reduction by NADPH requiring flavoprotein oxidoreductases such as cytochrome P450 reductase as has been previously shown (Bachur *et al.*, 1979). These enzymes have been shown to be present in mammalian S9 fraction (Beaune and Guengerich, 1988) and MCF-7 cells (Sinha *et al.*, 1987). Other enzymes are also present in MCF-7 S9 fraction which have been shown to metabolically activate doxorubicin. These include xanthine oxidase (Bates and Winterbourn, 1982; and section 1.8.2) NADH-dehydrogenase (Haidle and Hunter McKinney, 1985) and NADH cytochrome b_5 reductase (reviewed by Powis, 1989). One electron reduction of doxorubicin results in formation of a semiquinone free radical which is transient under aerobic conditions because it reacts with molecular oxygen at a diffusion controlled rate ($3.0 + 0.2 \times 10^8 M^{-1}s^{-1}$, Butler and Hoey, 1987) resulting in formation of superoxide anions. Formation of superoxide anions can lead to a cell damaging free radical cascade beginning with dismutation of the superoxide anion either spontaneously [$5x10^5 M^{-1}s^{-1}$] or enzymically [$2x10^9 M^{-1}s^{-1}$] by superoxide dismutase [SOD] [Cu-Zn SOD present in the cytoplasm or Mn-SOD in the mitochondrial matrix (see section 1.10.1) [equation (1)]. The hydrogen peroxide resulting from dismutation of superoxide anions, in the presence of trace amounts of transition metals, can lead to the formation of hydroxyl radicals [equation (2)] via the iron catalysed Haber-Weiss reaction. As discussed in section 1.9 hydroxyl radicals are highly reactive species and will damage DNA, proteins and lipids. The formation of reactive oxygen species in NADPH fortified MCF-7 S9 fraction mediated by doxorubicin was investigated using the technique of spin trapping [section 1.12.3]. No DMPO-superoxide anion spin adduct [DMPO-OOH] formation was observed in this system which appears contradictory to the results obtained using biochemical indicators of superoxide anion formation. However, a DMPO-hydroxyl radical [DMPO-OH] spin adduct was observed in this system [figure 2.7]. This is because the superoxide anion reacts very slowly with DMPO ($10\ M^{-1}s^{-1}$) whereas it

dismutates [equation (1)] spontaneously at $5x10^5 M^{-1}s^{-1}$ and enzymically at $2x10^9 M^{-1}s^{-1}$ [reviewed by Pou *et al.*, 1989]. Hydrogen peroxide can be further reduced to form hydroxyl radicals by the iron catalysed Haber-weiss reaction [equation (2)].

$$HO_2\cdot + O_2^- \xrightarrow{+H^+} H_2O_2 + O_2 \qquad (1)$$

$$M^{2+} + H_2O_2 \longrightarrow M^{3+} + OH^- + OH\cdot \qquad (2)$$

where M is a transition metal [see section 1.8.3]. The metal ion may be reoxidised by the superoxide anion [equation (3)] or by the semiquinone radical under anaerobic conditions [equation (4)] (reviewed by Powis, 1989).

$$M^{3+} + O_2^- \rightleftharpoons M^{2+} + O_2 \qquad (3)$$

$$M^{3+} + QH\cdot \underset{-H}{\rightleftharpoons} M^{2+} + Q \qquad (4)$$

The hydroxyl radical reacts much faster than the superoxide anion with DMPO ($2x10^9 M^{-1}s^{-1}$) [reviewed by Pou *et al.*, 1989]. Furthermore, the DMPO-OOH adduct formed by superoxide anions is rapidly converted to DMPO-OH either chemically or enzymatically [reviewed by Pou *et al.*, 1989] (eg by glutathione peroxidase, Samuni *et al.*, 1989). This may explain why in the present study a DMPO-superoxide anion spin adduct was not observed. It was necessary in the present study to ensure that DMPO-OH formation was not due to decomposition of DMPO-OOH. This was achieved by the use of catalase (300 units) which decomposes hydrogen peroxide (section 1.10.1) and DMSO (16mM) which scavenges hydroxyl radicals (reviewed by Pou *et al.*, 1989). Inhibition of doxorubicin mediated DMPO-OH formation in NADPH-fortified MCF-7 S9 fraction in the presence of these scavengers indicates that hydroxyl radical formation was responsible for the DMPO-OH adduct observed [see figure 2.7]. At

higher DMSO concentrations (200mM) a spin adduct characteristic of the DMPO-methyl radical [DMPO-CH_3] spin adduct was produced in a system of doxorubicin and NADPH-fortified MCF-7 S9 cell fraction (figure 2.8). This is formed by the reaction of hydroxyl radical with DMSO to generate a methyl radical, which reacts with DMPO to give a DMPO-CH_3 adduct (see figure 2.26). This provides further evidence for hydroxyl radical formation in NADPH-fortified MCF-7 cell S9 fraction in the presence of doxorubicin. During spin trapping studies a small DMPO-OH adduct signal was observed even in the absence of drug or S9 fraction [figure 2.8]. This was likely to be due to either O_2 or trace iron mediated oxidation of DMPO. The presence of the iron chelator desferrioxamine in the incubate should have cancelled the effect of iron on DMPO. However, desferral has recently been shown to form a nitroxide free radical species in the presence of a hydroxyl radical generating system (Morehouse *et al.*, 1987) and systems that generate superoxide anions (Davies *et al.*, 1987; Li *et al.*, 1989). In summary, these spin trapping studies support the redox cycling of doxorubicin in MCF-7 S9 cell fraction giving rise to the highly reactive hydroxyl radical. Spin trapping was also used to investigate redox cycling by doxorubicin in intact viable MCF-7 human breast cancer cells. As indicated by the formation of doxorubicin free radical species (figure 2.6) [as discussed above], formation of a catalase and DMSO sensitive DMPO-OH adduct (figure 2.9) and doxorubicin concentration dependent stimulation of oxygen consumption (figure 2.10) in an MCF-7 cell suspension, redox cycling would appear to be occurring. By analogy to the results obtained in MCF-7 S9 cell fraction it is likely that a doxorubicin semiquinone is being formed enzymatically which consumes molecular oxygen by reducing it to superoxide anions. Formation of this species is likely to give rise to the hydroxyl radical by the reactions previously described [equations (1-3)]. The findings of the present study are supported by the work of other researchers who have investigated free radical formation by doxorubicin in tumour cells. Doxorubicin has been shown to stimulate oxygen consumption and form a semiquinone free radical species in Chinese hamster ovary cells (Keizer *et al.*, 1988), rat hepatocytes (Lavelle *et al.*, 1985) and Hela cells (Partridge, 1987). Daunomycin has also been shown to generate a semiquinone free radical in rat hepatocytes, yoshida ascites cells and Ehrlich ascites cells (Bozzi *et al.*, 1981). Sato *et al.* (1977) and

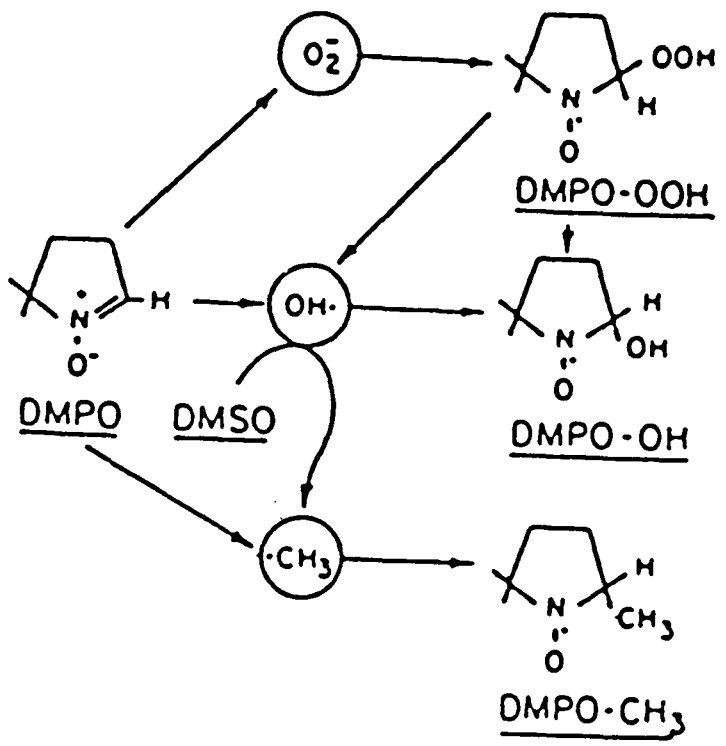


Figure 2.26 *Diagram of DMPO spin-trapped adduct formation following the interaction of DMPO with superoxide and hydroxyl radical in the presence of DMSO.*

Doroshow (1986a and 1986b) have shown that Ehrlich ascites tumour cells and MCF-7 human breast cancer cells, in the presence of doxorubicin, generated increased amounts of reactive oxygen species as determined using spin trapping. Also, using esr spin trapping techniques Sinha *et al.* (1987a and 1987b) demonstrated that doxorubicin increased hydroxyl radical formation in doxorubicin sensitive MCF-7 cells. Dusre *et al.* (1989) showed that buthionine sulphoxamine (BSO), an inhibitor of glutathione synthesis, potentiated doxorubicin mediated DMPO-hydroxyl radical spin adduct formation and cytotoxicity in doxorubicin-resistant MCF-7 cells. Indirect studies using doxorubicin resistant tumour cell lines have indicated doxorubicin redox cycling to be important in mediating cell kill [see section 1.16.4.4]. Moreover, modulation of cellular protection systems against reactive oxygen formation has been found to influence doxorubicin cytotoxicity, indicating a role for redox cycling in the mechanism of action of doxorubicin [see section 1.16.4.1]. However, the studies carried out by other workers have not systematically investigated each line of evidence for doxorubicin redox cycling in a cell line as has been undertaken in the present study.

In the present study the DMPO-OH adduct produced in viable MCF-7 cells in the presence of doxorubicin appeared to be located extracellularly, as indicated by the diminuition of this signal in the presence of catalase, an enzyme which is unlikely to cross the cell membrane. In addition, persistance of the esr signal in the cellular supernatant after the cells have been removed indicates an extracellular site of hydroxyl radical formation. DMPO is lipid soluble so it is capable of permeating the cell membrane (Pou *et al.*, 1989). This finding would appear to agree with the proposal of Tritton *et al.* (1983) that doxorubicin can be metabolically activated enzymatically at the cell surface and does not need to enter the cell to express cytotoxicity. Sinha *et al.* (1987b) also showed that 60-65% of the DMPO-OH adduct was produced extracellularly in doxorubicin treated MCF-7 cells as indicated by the use of chromium oxalate as a line broadening agent. Furthermore SOD inhibited DMPO-OH formation and exogenous NADPH stimulated hydroxyl radical formation. This might be explained if the DMPO-OH adduct or hydrogen peroxide produced during doxorubicin redox cycling diffused out of the cell. Furthermore, despite its lipophilicity, DMPO has been

proposed by Samuni *et al.* (1989a) to be unsuitable for use for spin trapping in cellular systems due to its rapid metabolism. As discussed previously DMPO-OOH can decompose to DMPO-OH and other products chemically or by enzymes such as glutathione peroxidase (reviewed by Pou *et al.*, 1989). Recent evidence has shown that DMPO-OH is degraded by superoxide anions (Samuni *et al.*, 1989b). From the results of the present study it is clear that hydroxyl radicals are produced in MCF-7 cells when they are treated with doxorubicin. However, due to the difficulties associated with intracellular spin trapping studies it is not possible to locate the site of doxorubicin activation in MCF-7 cells.

The formation of reactive oxygen species by doxorubicin redox cycling in MCF-7 cell fraction and whole cells as determined in the present study would appear to correlate with similar studies in MCF-7 human breast cancer cells and other cell lines. Doxorubicin redox cycling would appear to occur in tumour cells with production of the potentially damaging hydroxyl radical. The overall consequence of this will ultimately depend on the site of generation of this reactive species and how well the cell protective systems (section 1.10) can cope with this oxidative insult. Possible biological end points of doxorubicin redox cycling in MCF-7 cells, namely DNA damage and cytotoxicity and are investigated in chapters 3 and 4. The findings reported in this section have also confirmed that the MCF-7 cell line has the enzymes required to mediate metabolic reductive activation of doxorubicin in the process of redox cycling. Therefore, this cell line appears suitable for investigation of redox cycling by other quinone containing antitumour agents.

2.4.2 *Comparison of redox cycling by mitozantrone, CI-941 and doxorubicin in MCF-7 cells*

Mitozantrone and CI941 are structurally related antitumour agents which have been developed in order to produce agents with reduced cardiotoxicity in comparison to doxorubicin. As described previously

their structural features were designed to favour DNA intercalation but reduce their interaction with flavin reductase enzymes and hence reduce their ability to undergo redox cycling, the mechanism proposed to mediate doxorubicin cardiotoxicity [see section 1.16.5]. Studies have been carried out to compare doxorubicin with mitozantrone and CI941 redox cycling in both animal and human tissues [Basra *et al.*, 1985; Graham *et al.*, 1987). However, no studies have yet been reported on the relative propensity of these agents to redox cycle in human tumour tissue.

Mitozantrone and CI941 inhibited base rate NADPH oxidation, produced negligible superoxide anion formation, did not form semiquinone free radicals and did not generate hydroxyl radicals in MCF-7 cell S9 cell fraction [tables 2.1 and 2.2; figures 2.11, 2.12 and 2.13]. This indicates that these compounds have little propensity to undergo redox cycling in MCF-7 cells in comparison to doxorubicin. Tables 2.4a and 2.4b compare NADPH oxidation and superoxide anion formation by these three compounds. Basra *et al.* (1985) found that mitozantrone stimulated base rate NADPH oxidation, superoxide formation and generated an esr signal attributed to semiquinone formation in human liver microsomal tissue, but at considerably lower levels than doxorubicin. Similar findings were found in mouse and rat liver and heart tissue (Basra, 1986). However, Vile and Winterbourn (1989) found that mitozantrone did not stimulate superoxide anion formation in rat liver microsomes. CI941 has also been found to have reduced propensity for metabolic activation in rat tissue compared to doxorubicin, no free radical being detected by esr (Graham *et al.*, 1987). Consistent with the inhibition of NADPH oxidation in this study, mitozantrone has also been shown to inhibit base rate NADPH dependent lipid peroxidation in rabbit liver and heart tissue (Kharasch and Novak, 1983a and 1985) and inhibit doxorubicin stimulated lipid peroxidation and superoxide formation. Similar findings were reported for CI941 in rat tissues (Graham *et al.*, 1987). In addition mitozantrone did not initiate lipid peroxidation in mouse liver and heart fractions (Patterson *et al.*, 1983) or rat liver microsomes (Vile and Winterbourn, 1989). The absence of enzymic reduction of mitozantrone and CI941 compared to doxorubicin in the present study correlates well with their one-electron reduction

Table 2.4a *Comparison of the effect of doxorubicin, mitozantrone and CI941 on NADPH oxidation in MCF-7 S9 fraction.*

Drug [100uM]	NADPH oxidation pmole $min^{-1}mg^{-1}$ S9 protein
Base	72.0 + 18.0
mitozantrone	49.7 + 2.2
CI941	48.96 + 2.9
doxorubicin	830.74 + 0.1

Table 2.4b *Comparison of superoxide anion formation in MCF-7 S9 fraction by mitozantrone , CI941 and doxorubicin.*

Drug [100μM]	Reduction of acetylated cytochrome c nmole $min^{-1}mg^{-1}$ S9 protein	adrenochrome formation nmole^{1}min^{1}mg S9 protein
mitozantrone	0.98 + 2.09	6.93+0.51*
CI941	1.71 + 0.95	4.42 +1.9*
doxorubicin	7.54 + 1.9*	42.0 +8.7*

Values represent mean + sd of three determinations.
* ($p<0.01$)

potentials at pH7.0 (E^1_7) [see table 2.5]. Both mitozantrone (E^1_7; -527+5mV) and CI941 (E^1_7; -538+10mV{Graham *et al.*, 1987}) have considerably more negative reduction potentials than doxorubicin (E^1_7; -328+5mV). However, the rates for the reaction of the respective semiquinones with molecular oxygen are all very fast [table 2.5]. As described previously [section 1.8.1] subsequent reactions of the superoxide anion are very rapid. This indicates that the rate limiting step for the activation of these agents and formation of reactive oxygen species is the step involving one electron enzymic reduction. The more negative reduction potentials of mitozantrone and CI941 means they are more difficult to reduce than doxorubicin. Furthermore, they are also likely to be poorer substrates for reductases than doxorubicin due to steric hinderance between these compounds and the flavin component of the reductase, the proposed site of interaction of quinone containing antitumour agents. Flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) mediate electron transfer from NADPH to cytochrome P450 (Vermilion, *et al.*, 1981). Both doxorubicin and mitozantrone have been shown to bind to FMN and FAD with high affinity, an interaction that has been proposed to be involved in electron transfer from the reductase to quinone containing drugs (Kharasch and Novak, 1981 and 1983c). In addition, the MCF-7 S9 cell fractions used in this study had lower NADPH cytochrome P450 reductase activity [1.19 nmoles min^{-1} mg^{-1} S9 protein] than human liver [166.1+49.4 nmoles $min^{-1}mg^{-1}$ protein] and mouse liver microsomes [222.3+4 nmoles $min^{-1}mg^{-1}$ protein] (figures indicate rate of reduction of cytochrome c, derived from Basra *et al.*, 1985). The cytosolic enzyme xanthine oxidase was found not to be involved in activation of mitozantrone or CI941 [see section 3.3.1.2]. The lack of detectable radical formation in the case of CI941 and mitozantrone can therefore be explained by a very low rate of semiquinone formation. The rate of doxorubicin semiquinone formation is typically measured as nmole NADPH oxidised min^{-1}, this will rapidly react with oxygen ($3.0x10^8 M^{-1s}$-1) to produce superoxide anions which will rapidly dismutate ($2x10^9 M^{-1}s^{-1}$) in the presence of SOD or spontaneously ($5x10^5 M^{-1}s^{-1}$) [see section 1.8.1]. From the above reaction rates it can be seen that high concentrations of semiquinone free radical need to be produced before it can be detected. For example, the lowest doxorubicin concentration at which semiquinone formation detected in MCF-7 S9 fraction was 50uM (figure 2.4).

Table 2.5 *Reduction potentials and rates of reaction for the semiquinone free radical [AQ ·] of quinone antitumour agents.*

Compound	Reduction potential mV	Reaction $AQ^{\cdot}/O_2$ $M^{-1}s^{-1}$	Reaction $AQ^{\cdot}/AQ^{\cdot}$ $M^{-1}s^{-1}$
doxorubicin	-328+5	$3.0+0.2\times10^{8}$	nd
mitozantrone	-527+5	$*5.1+0.5\times10^{8}$	$2.74+1.0\times10^{9}$
CI941	-538+10	$*1.8+0.1\times10^{9}$	nd
1AQ	-469+8	$1.9+0.1\times10^{9}$	$1.6+0.8\times10^{9}$
1,4AQ	nd	nd	nd
1,5AQ	-495+5	$2.0+0.15\times10^{9}$	$1.5+0.6\times10^{9}$
1,8AQ	-497+8	$1.9+0.1\times10^{9}$	$1.9+0.3\times10^{9}$

Derived from Basra (1986) except *derived from Butler and Hoey (1987)
nd=not determined

The main concern about the drug concentrations used in these assays is as to whether these concentrations are physiologically relevant. Basra *et al.* (1985) suggest that despite the peak plasma concentration of doxorubicin and mitozantrone being approximately 1uM, it is likely that intracellularly higher local concentrations are attained due to the high binding affinities of these drugs for cellular membranes and DNA (reviewed by Gianni *et al.*, 1983). Given that the DNA binding affinities of the quinone antitumour agents are in the region of 10^6-10^7 (see table 3.3) the nuclear concentration of these agents could be 10^6 fold higher than other regions of the cell.

2.4.3 *Redox cycling by alkylaminoanthraquinone antitumour agents*

A series of hydroxyethylaminoethylamino substituted anthraquinones with structures based on the requirements for DNA intercalation by doxorubicin and mitozantrone were synthesised by Murdock *et al.* (1979) as part of the screening programme to find less cardiotoxic antitumour agents and later by Gandecha (1985). These compounds (figure 1.35) have identical side chains to mitozantrone but do not have the aromatic hydroxyl groups on the A ring of the anthraquinone chromophore. By analogy with doxorubicin, due to the possession of the anthraquinone moiety these compounds are candidates for metabolic activation by flavin reductase enzyme systems [see section 1.16.4.1]. Therefore the ability of these compounds to undergo redox cycling in the MCF-7 human breast cancer cell line was investigated in this study [see section 2.3.3.4].

All four AQ's showed some evidence for redox cycling in MCF-7 S9 cell fractions. The 1AQ strongly stimulated NADPH oxidation [figure 2.15], superoxide anion formation [figure 2.17] and the formation of hydroxyl radicals [figure 2.19] in this system. This provides evidence for the occurrence of a redox cycling process involving flavin reductase one electron reduction to a semiquinone free radical intermediate as previously described for doxorubicin [section 2.4.1]. However, no esr signal could be detected for 1AQ to indicate drug semiquinone formation [figure 2.18]. As described in section 2.4.2, this is likely to be due to the slow rate of formation of this species enzymically and its

sensitivity to the presence of residual oxygen. The 1,8AQ also stimulated NADPH oxidation [figure 2.14], superoxide anion formation [2.16], hydroxyl radical formation and produced a free radical species detectable by esr (figure 2.18). These results are consistent with this compound undergoing redox cycling in MCF-7 S9 cell fraction as described for doxorubicin [section 2.4.1]. 1,5AQ was found to have no significant effect on base rate NADPH oxidation (figure 2.14) and produced negligible superoxide anion formation (figure 2.16) suggesting only a small level of enzymic activation of this compound. However, these results are contradicted by the detection of a free radical species for 1,5AQ by esr in MCF-7 cell S9 fraction, consistent with semiquinone formation (figure 2.18). Furthermore 1,5AQ also generated hydroxyl radicals in MCF-7 cell S9 fraction [figure 2.21]. This would suggest that despite 1,5AQ having no significant effect on base rate NADPH oxidation that the 1,5AQ is possibly being activated by a non-NADPH requiring enzyme system. However, 1,5AQ was found not to be activated by the cytosolic enzyme xanthine oxidase [see section 3.3.1.3]. Finally 1,4AQ also stimulated NADPH oxidation in MCF-7 S9 fraction (figure 2.14) but formed negligible superoxide anions (figure 2.16) as determined by the reduction of acetylated cytochrome c. However, no hydroxyl radical formation or drug free radical species could be detected in MCF-7 cell S9 fraction for this compound (figures 2.18 and 2.20). This indicates that in contrast to the other AQ's this compound does not redox cycle in the MCF-7 cell line.

The weak semiquinone free radical esr signals obtained for the AQ's in MCF-7 S9 cell fraction (figure 2.18) can be explained by the slow rate of enzymic formation of the one electron reduced drug intermediate compared to the rate of its decay [as discussed in section 2.4.2]. A strong esr signal could be consistently obtained for doxorubicin semiquinone in an identical system [figure 2.4]. Considering the levels of NADPH oxidation and superoxide formation produced by 1AQ and 1,8AQ which are of similar level to that stimulated by doxorubicin it is surprising that measurable esr signals could not be obtained for the semiquinones of these compounds. However, due to AQ's having more negative reduction potentials [table 2.5] than doxorubicin they are likely to be more difficult to reduce by reductases and are therefore

likely to be reduced more slowly. These reduction potentials are still sufficiently negative to reduce molecular oxygen to the superoxide anion [reduction potential for 1 Molar O_2/O_2^- = -155mV]. In addition, the reaction rates for the interaction of the AQ semiquinones with oxygen [see table 2.5, Basra, 1985] are approximately six times faster than that obtained for doxorubicin [$0.3+0.2 \times 10^9 M^{-1}s^{-1}$]. The esr signal intensity is dependent on the equilibrium between rate of formation and rate of decay of the semiquinone. This means in the presence of residual oxygen the semiquinone is not likely to build up in intensity due to its slow rate of formation and fast rate of decay. Contributing to the rate of decay of this species is the interaction of two semiquinone radicals [reaction rates table 2.5] (equation 1) which for anthrasemiquinones is a diffusion controlled process. This mechanism for the loss of semiquinone will predominate under anaerobic conditions. However the semiquinone free radical may be reformed by comproportionation if sufficient fully reduced quinone is present (hydroquinone) [equation 2].

$$AQH^{\cdot} + AQH^{\cdot} \rightleftharpoons AQH_2 + AQ \quad (1)$$

$$AQH_2 + AQ \rightleftharpoons 2AQH^{\cdot} \quad (2)$$

Considering the five parameters of redox cycling investigated in this study a rank order of propensity to undergo this process can be constructed for the AQ's:- 1AQ = 1,8AQ > 1,5AQ > 1,4AQ. Overall, it would appear that doxorubicin has a greater propensity than 1AQ, 1,5AQ and 1,8AQ to redox cycle whereas mitozantrone, CI941 and 1,4AQ do not redox cycle in MCF-7 cells. The results obtained in this study are consistant with the previous findings of Basra (1986) who studied redox cycling by AQ's in mouse liver microsomes. In these studies 1,8AQ was found to be the most active redox cycling agent and 1,4AQ the least active with 1AQ and 1,5AQ being intermediate between these two compounds. However, it is unrealistic to directly compare the results of the present study in MCF-7 cells with those obtained from mouse liver microsomes. 1,4AQ, (also known as ametantrone) has been found to behave in a similar manner to mitozantrone in several systems [see section

2.4.2]. Kharasch and Novak (1982, 1983a and 1983b) found 1,4AQ inhibited base rate lipid peroxidation and doxorubicin-stimulated lipid peroxidation in rat and rabbit liver microsomes, cardiac sarcosomes and cardiac mitochondria. This effect was proposed to be due to an inhibition of hydroperoxide dependent initiation and propagation reactions (Kharasch and Novak, 1985). In addition, in rabbit liver microsomes ametantrone did not significantly stimulate basal rate NADPH oxidation or superoxide anion formation (Novak and Kharasch, 1985). These studies support the absence of redox cycling observed with the 1,4AQ in the present study.

It would appear that structural alteration of the AQ's by changing the position of the side chains on the anthraquinone chromophore [see figure 1.35] influences the stimulation of NADPH oxidation, superoxide anion formation, hydroxyl radical formation and formation of a drug free radical species in MCF-7 S9 cell fraction indicating differences in the abilty of the AQ's to undergo reductive metabolic activation. This may be due the positioning of the side chains sterically hindering interaction of the anthraquinone chromophore with the flavin component of reductase enzymes (as discussed in section 2.4.2).

In summary, evidence has been accumulated that doxorubicin undergoes a redox cycling process in MCF-7 human breast cancer cells with resultant production of the potentially cell damaging hydroxyl radical. In contrast, mitozantrone and CI941 do not redox cycle in this cell line. Furthermore, a series of alkylaminoanthraquinones based on mitozantrone have also been shown to redox cycle in MCF-7 S9 cell fractions to varying degrees depending on the position of the hydroxyethyl-aminoethylamino side chains on the anthraquinone moiety. In subsequent chapters, biological end points which are possibly caused by redox cycling and resultant reactive oxygen formation by the quinone antitumour agents of the present study are assessed in the MCF-7 cell line.

CHAPTER 3

Studies on the effects of quinone antitumour agents on DNA

CHAPTER 3

Studies on the effects of quinone antitumour agents on DNA

3.1 Introduction

As described in sections 1.16.1 and 1.17 anthracycline and anthraquinone antitumour agents bind to DNA by a mixture of intercalative binding and electrostatic interaction with the surface of the DNA helix. Besides this characteristic property, chapter 2 has shown that the quinone antitumour agents of the present study have varying propensities to undergo metabolic reductive activation during reductase mediated redox cycling, resulting ultimately in hydroxyl radical formation. This chapter investigates several aspects of DNA interaction by the antitumour agents of the present study:-

1. The ability of these agents to produce DNA damage in a system containing purified reductases and plasmid DNA.

2. The ability of these agents to mediate plasmid DNA strand breakage in the presence of iron and reduced glutathione.

3. The effect of these agents on topoisomerase activity in MCF-7 cell nuclear extract.

4. The ability of these agents to produce DNA strand breakage in MCF-7 cells *in vitro*.

3.2 Methods

3.2.1 *Determination of the effects of quinone antitumour agents on plasmid DNA in the presence of purified NAD(P)H dependent reductases.*

This assay was based on the strand breakage of plasmid pBR322 DNA which is supercoiled in its native form. Nicking of the DNA results in conversion of the supercoiled [form I] DNA to relaxed [form II] DNA or linear [form III] DNA (see section 1.12.3 for full details). Open circular and linear DNA present after incubation of the native supercoiled DNA with drug/enzyme were separated by submarine agarose gel electrophoresis. The DNA species were quantitated by staining with ethidium bromide, photographing the gel [using UV transillumination] and determining the intensity of the DNA bands on the negative by laser densitometry. Appendix figure A3 shows that the DNA concentration used was in the linear region of a plot of DNA concentration against band intensity, which enabled maximum sensitivity for detection of changes in DNA concentration.

The components of each incubate were dispensed into plastic eppendorf tubes (2ml) in the following order to produce a total volume of 100ul: Tris buffer [100mM, pH8.0], $MgCl_2$ [3mM], NADH [0.4mM] or NADPH [1mM], xanthine oxidase [2.5 units] or cytochrome P450 reductase [23.4 units], drug [10-100uM] and finally plasmid pBR322 DNA [300ng]. Plasmid DNA was added last to minimise DNA damage during the preparation of each incubate. The contents of the control and test tubes are summarised in table 3.1.

On addition of the plasmid DNA, the tubes were capped and mixed by centrifugation for five seconds in a microcentrifuge [Hema-C, Jouan] and then incubated in the dark at 37C for thirty minutes. At the end of the incubation, NaCl [5M, 100ul] was added to each tube, following which the drug was extracted using water-saturated n-butanol [200ul aliquots].

Table 3.1 *Experimental table for a typical plasmid assay*

component	control A	control	control C	experimental
$MgCl_2$	+	+	+	+
enzyme	-	+	-	+
tris pH 8	+	+	+	+
NAD(P)H	-	+	+	+
drug	-	-	+	+
DNA	+	+	+	+

control A = DNA only, control B = DNA + enzyme and
control C = DNA + drug

Upon addition of the solvent the tubes were gently agitated to mix, then spun in a microcentrifuge for five seconds and the n-butanol layer removed and discarded. This procedure was repeated until drug colouration of the aqueous phase was no longer evident [2-4 extractions].

In order to precipitate the DNA, pre-chilled ethanol [-20C] was added to the samples and the tubes placed in a freezer (-20C) for one hour. Following this the samples were spun [12,000g, 10min] on a microcentrifuge to pellet the DNA. The caps of the tubes were cut off and the ethanol decanted carefully so as to avoid disturbing the DNA pellet. The tubes were then inverted and any remaining ethanol removed using a capillary tube. The samples were then dried by placing under a vacuum for five minutes. Following drying, TE buffer [Tris-HCl (10mM) EDTA (1mM), 5ul, pH8] was added and the bottom of the tubes 'flicked' once to dislodge the DNA pellet. The tubes were centrifuged for five seconds and then incubated at 37C for fifteen minutes to redissolve the DNA pellet. Following incubation, loading buffer [bromophenol blue (0.25%), sucrose (1.17M), 5ul] was added to the samples and the tubes centrifuged (12,000g, 5 seconds) to mix. Aliquots (10ul) of the samples were loaded onto a pre-prepared agarose gel [agarose (1%) in Tris base (39.6mM), glacial acetic acid (18.3mM), EDTA (1mM), (TAE buffer)] containing 16 sample wells (see appendix A4). The gel was placed in an electrophoresis tank and submerged in electrophoresis buffer [TAE] and twenty volts [35 mAmps] applied overnight (or 150V for one hour), the DNA moving in the direction of the anode.

After electrophoresis the gel plate was placed in ethidium bromide [2ug/ml, in TAE] for fifteen minutes in order to stain the DNA, excess stain being removed by placing the gel plate in destaining buffer [TAE] for thirty minutes. The gel was then photographed.

Photography was carried out using a Land camera [MP-4, Kodak] by illuminating the gel from underneath with uv light at 300nm using a transilluminator [UVP inc]. The film used was Kodak professional (Pelica T max 100 4052), with an exposure time of twenty seconds, two

orange filters in series (Wratten orange filter 23A) being used to enhance the contrast. The negative was developed in total darkness using developer [ID11, Ilford] (time of developing dependent on temperature of developer) and fixed for seventeen minutes using fixer [Hypam, Ilford].

Quantitation of negatives was carried out using a Ultroscan laser densitometer [Model 2202, LKB Bromma] with Gelscan interface and software package [LKB 2190-001] run on an Apple II computer. The accumulated data was analysed using the programme 'Gelscan' which presented the results in the form of densities and relative percentages of the DNA species in each sample. The recorded density of supercoiled plasmid DNA was corrected at this stage using the correction factor of 1.22, since ethidium bromide has reduced binding affinity for this form of DNA [Hertzberg and Dervan, 1982].

3.2.2 *Determination of the effect of quinone antitumour agent iron complexes on plasmid DNA in the presence of reduced glutathione.*

3.2.2.1 *Preparation of anthraquinone-iron complexes*

Complexes between iron and doxorubicin, CI941 or mitozantrone were prepared as a 2:1 iron:drug ratio in the following manner.

Drug [3mM], in HCl [pH 2.0] was mixed with ferrous ammonium sulphate [3mM] in HCl [pH 2.0] in a 1:2 ratio. The mixture was diluted (1:5) using tris buffer [200mM, pH 7.4] to give a final concentration of 0.2mM drug: 0.4mM iron(II) at pH 7.4. Solutions containing iron or drug only were prepared for use as controls by replacing either ferrous ammonium sulphate or drug with HCl [pH 2.0] in the above protocol. Anthrapyrazole-ferric iron complexes were prepared in an identical manner using ferric chloride.

3.2.2.2 *Plasmid assay*

This assay was essentially the same as that described above [section 3.2.1] except the incubate contained the following:
Tris buffer [100mM, pH7], glutathione (reduced form) [25mM, in Tris buffer], drug-iron complex [0.5-50uM, 1:2 drug:iron ratio] and DNA [300ng]. The controls consisted of samples containing iron (ferrous ammonium sulphate, 2-100uM) or drug (0.5-50uM).

To aid the extraction of the drug iron complex from the DNA desferrioxamine [5mM, 5ul] was added prior to extraction with water-saturated n-butanol.

3.2.3 Determination of the effect of quinone antitumour agents on MCF-7 cell topoisomerase activity

This assay involved the use of supercoiled [form I] pBR322 plasmid DNA as a substrate for a topoisomerase containing nuclear extract from MCF-7 cells. Topoisomerase I and II activity can be separated by including adenosine 5'-triphosphate (ATP), which is required for topoisomerase II activity only (reviewed by Wang, 1985). In this system topoisomerase activity unwinds or relaxes the supercoiled DNA producing various relaxed DNA forms or topomers including open circular (form II) DNA. The DNA species are resolved by submarine gel electrophoresis. Two types of drug effect on topoisomerase activity can be determined in this assay system:-

a) Inhibition of the relaxing activity of topoisomerase

b) Formation of topoisomerase cleavable complex by the presence of drug. The cleavable complex consists of an enzyme complex 'trapped' on the DNA. On treatment of this complex with proteinase K, single or double strand breaks are revealed. Thus, in this assay drug-mediated cleavable complex formation would be indicated by the

presence of open circular or linear DNA (see section 1.12.3).

3.2.3.1 *Preparation of a crude nuclear extract from MCF-7 cells*

This was prepared using the method described by Minford *et al.* (1986). Firstly MCF-7 cells [10^7-10^9] in exponential growth phase were harvested and suspended in nucleus buffer [$MgCl_2$ (5mM), KH_2PO_4 (1mM), EGTA (1mM), dithiothreitol (0.1mM), glycerol (10%), NaCl (0.15M), pH 6.5]. The cells were washed twice in nucleus buffer by centrifuging [10min, 1200rpm, 4C] using a refrigerated bench centrifuge [CR122, Jouan]. In order to permeabilise the cells the cell pellet was resuspended in nucleus buffer (10ml, 4C) and the volume made up to 100ml with lysis buffer [$MgCl_2$ (5mM), KH_2PO_4 (1mM), EGTA (1mM), dithiothreitol (0.1mM), glycerol (10%), triton X-100 (0.35%) and phenylmethanesulphonyl fluoride (0.1mM), pH 6.5]. The cell suspension was rotated at low speed [15min, 4C] using a mixing device [Heidolph]. The suspension was then divided into two centrifuge tubes (50ml) and centrifuged [10min, 1400rpm, 4C] in order to spin down the released nuclei. The nuclear pellet was washed once with nucleus buffer by centrifuging [10min, 1400rpm, 4C] and the resulting pellet resuspended in extraction buffer [nucleus buffer containing NaCl (0.35M)]. The suspension was gently rotated [30min, 4C] in order to extract nuclear protein. The suspension was then centrifuged [20min, 1800rpm, 4C] and the resulting supernatant removed and further centrifuged [10min, 12,000g, 4C] in a microcentrifuge [Hema-C, Jouan]. The supernatant [nuclear extract] was stored in aliquots [100ul] under liquid nitrogen until required.

3.2.3.2 *Determination of topoisomerase relaxing activity in MCF-7 cell nuclear fraction*

The basic components of the incubate [100ul] were topoisomerase buffer [KCl (100mM), $MgCl_2$ (10mM), EDTA (0.5mM), BSA (30ug/ml) and dithiothreitol (5mM) in tris buffer (100mM), pH 7.4], nuclear extract

(1-15ug protein) and plasmid DNA (500ng). Each extract concentration was incubated in the presence or absence of ATP (1mM). Control incubates consisted of DNA only and DNA plus ATP. The incubation was carried out for 15-30 minutes at 37C, and the reaction terminated by the addition of sodium dodecyl sulphate [2%] and proteinase K (0.05mg)[20 Anson units/g, BDH], mixing by centrifugation [5sec] and incubating [30 min, 37C]. NaCl [5M, 100ul] and pre-chilled ethanol [400ul] were added to the incubates and the protocol described in section 3.2.1 was followed.

3.2.3.3 *Determination of the effect of quinone antitumour agents on topoisomerase DNA relaxing activity in MCF-7 cell nuclear extract*

The basic incubate was as described in section 3.2.3.2. Incubations were carried out with nuclear extract [1ug] in the presence or absence of drug and in the presence or absence of ATP [1mM]. Control incubate consisted of DNA only and DNA plus ATP. The incubation was carried out for fifteen minutes at 37C. The reaction was terminated by the addition of SDS [2%] followed by a further incubation [45min, 50C]. The protocol subsequently followed was as described in section 3.2.1.

3.2.3.4 *Determination of topoisomerase cleavable complex formation by quinone antitumour agents.*

This assay was carried out as described in section 3.2.3.3. However the reaction was terminated using SDS [2%] and proteinase K (0.05 or 0.1mg).

3.2.4 Determination of quinone antitumour agent induced DNA strand breakage in MCF-7 cells by alkaline elution.

This technique, developed by Kohn et al. (1976) utilises filters to determine DNA strand breaks in mammalian cells produced by cytotoxic drugs or radiation. The filters are used as a mechanical barrier to the passage of large DNA strands (see section 1.11.1). In this study this technique was used to determine DNA single strand breaks after treatment of MCF-7 cells with radiation or quinone antitumour agents.

3.2.4.1 *Preparation of filter assembly*

Firstly ten filter systems were assembled as follows:-
A polycarbonate membrane filter [0.8uM, 25mm diameter, Nucleopore (USA), obtained from Sterilin Ltd (UK)] was placed in a pre-cooled swinnex filter holder [25mm diameter, Millipore] fitted with a rubber gasket. The top male Luer connection of the swinnex was connected via a threeway tap [type 875.00, Vygon] to a syringe with a concentric luer lock connector [50ml, Fisons]. The bottom connector of the swinnex was connected to another threeway plastic tap [type 875.00, Vygon]. The filter assembly was clipped vertically onto an especially constructed rack which enabled drainage of the assembly into a perspex channel, leading to a waste container. Using a syringe (50ml), connected to a threeway tap, the syringe was filled with ice cold phosphate buffered saline (PBS) (10ml, pH7.2) via the bottom tap. The PBS was passed upwards and downwards through the swinnex several times whilst tapping the swinnex in order to remove all air bubbles. With the upper tap in the closed position, the syringe was filled from above with a further 40ml of ice cold PBS. The PBS was then allowed to drip through the filter assembly until the meniscus reached the apex of the syringe. The swinnex was left at this stage (1 hour) in order for the filter to cool prior to loading of cells.

3.2.4.2 *Treatment of cells*

a) Radiation

MCF-7 cells were harvested and dispersed as described in section to provide a concentration of 0.5-2.0 x 10^6 cells ml^{-1}. Aliquots (1ml) of cell suspension were placed in ten screw cap plastic vials (2ml, Nunc) and placed on ice. The vials were exposed to gamma radiation, by placing in a pre-cooled lead block [ave. thickness 35mm] and inserting this into an automated ^{60}Co gamma source (Vickrad). The vials were exposed to the gamma source for varying time lengths (46, 92, 184 secs) to give doses of radiation corresponding to 2, 4 and 8 Grays (Elsy, D., Leicester University, personal communication). Duplicate vials were treated at each dose, whilst dupicate vials were left untreated to serve as controls. After irradiation the vials were immediately placed on ice to prevent DNA repair (Kohn *et al.*, 1981). The cells were then ready for lysis.

b) Antitumour agents

MCF-7 cells were harvested and dispersed as described in appendix A1.2 and section 4.2.1, suspended in RPMI-1640 growth medium at a concentration of $5x10^6ml^{-1}$. Aliquots of cell suspension (1ml) were placed in plastic centrifuge tubes (12ml, Sterilin) and transferred to a water bath (37C). A volume of drug solution was added to the cell suspension so as to give the desired final drug concentration. The cells were incubated with drug for one hour, following which the cells centrifuged (900rpm, 10min, 4C) using a refrigerated bench centrifuge (CR422, Jouan). The drug supernatant was discarded and the cell pellet resuspended in ice cold PBS (2ml). The cells were centrifuged as above to wash the cells free of drug. Finally, the pellet was resuspended in ice cold PBS (1ml) and placed on ice. The cells were then ready for lysis.

3.2.4.3 *Loading and lysis of cells*

With the upper tap in the closed position the syringe was filled with a further volume of ice cold PBS (40ml). MCF-7 cells ($0.5x10^6$, in 1ml PBS) were mixed with the PBS in the syringe. The cells were loaded onto the filter by allowing the suspension to drip through the swinnex until the meniscus reached the apex of the syringe. The cells on the filter were then washed with a further aliquot of PBS (20ml). Lysis solution (sodium dodecyl sarkosine (sodium sarkosyl) (0.2%), disodium EDTA (0.04M), NaCl (2M), pH10, 2ml) containing proteinase K [BDH, 0.01 Anson units ml^{-1} (0.5mg ml-1)] was placed in the syringe and allowed to drip through into the swinnex until the meniscus reached the apex of the syringe. At this point the filter assemblies were covered with foil to protect the DNA from light mediated strand breakage. The cells were left in contact with the lysis solution for one hour to allow complete digestion of cellular and DNA associated protein. After one hour cellular debris, remaining protein and RNA was washed through the filter with an EDTA wash solution (tetrasodium EDTA (0.02M), pH10.0, 10ml). The EDTA wash was allowed to reach the apex of the syringe. At this point the filters could be left for up to one hour before elution of the DNA was started.

3.2.4.4 *Elution of DNA*

Once the EDTA wash was complete the lower taps of the filter assemblies were connected via a needle (19 gauge, stainless steel, with sawn off point, BDH) and transmission tubing (0.02mm internal diameter, Chem Lab) to a ten channel peristaltic pump (HR flow inducer type MHRE 300, Watson Marlowe Ltd) set to give a flow rate of 0.035ml min^{-1}. The collection side of the pump led, via transmission tubing (0.02mm), to a ten channel fraction collector (modified Minirac fraction collector, LKB) set to collect eleven fractions per channel at fortyfive minute intervals in graduated sample cups (4ml, Chem Lab).

When the fraction collecting apparatus was assembled, single strand eluting solution [EDTA free acid (0.02M), Tetraethyl ammonium hydroxide (0.1M), pH12.3, 25ml] was placed in the syringes with the upper tap closed. The taps were opened and the peristaltic pump switched on. When the eluted solution reached the first sample cups, the fraction collector was switched on. Elution of DNA was carried out with the filter assemblies protected from light by an aluminium foil covering.

3.2.4.5 *Treatment of filters*

When all the fractions were collected the swinnex and lower tap were disconnected from the syringe and upper tap and inverted onto a glass Universal vial (30ml). The transmission tubing connected to the fraction collector was disconnected, the end placed in a beaker of eluting solution and the peristaltic pump switched on [in reverse, 0.1ml min^{-1}] in order to flush through and wash the tubing. After thirty minutes the pump was switched off, and the filter assembly disconnected from the pump. The transmission tubing was immediately washed by pumping distilled water through it for one hour. The swinnex was tapped firmly on the neck of the vial to dislodge any residual DNA. The swinnex was then flushed through with eluting solution (5ml) [via a syringe from the top] and gently unscrewed above the vial so as to collect residual DNA. The filter was removed using a pair of fine tweezers and placed in the vial. The two halves of the swinnex and rubber gasket were thoroughly rinsed with a further aliquot of eluting solution (5ml) using a Pasteur pipette. Screw caps were placed on the vials and the vials transferred to a water bath (60C) for one hour to denature the remaining DNA. After one hour the vials were removed and the filter/eluting solution sonicated at full power for one minute using a sonic probe (Model KT100, Kerrys Ultrasonics Ltd).

3.2.4.6 *Determination of DNA content in eluted fractions*

Firstly a calibration graph using denatured [single stranded] calf

thymus DNA [see appendix A8.0] was prepared for the fluorescent dye binding assay. Denatured calf thymus DNA standards (0-10ug ml^{-1}) were prepared in eluting solution.

In order to neutralise the pH of each standard and quantitate the DNA present, Hoechst H33258 ($4.5x10^{-7}M$) in buffer solution [KH_2PO_4 (0.042M), potassium phosphate buffer (0.05M, pH 7.0), standard saline citrate (0.15M, pH 7.0), 2ml)] was added to DNA standard (1ml) in a quartz fluorimetric cuvette and mixed using a plastic spatula. The fluorescence of the sample was measured using a Luminescence spectrometer (LS-5, Perkin Elmer) with an excitation wavelength of 340nm, an emission wavelength of 440nm and excitation and emission slit widths of 10nm. Background fluorescence was subtracted from all readings and a calibration curve constructed (see section A15 for a typical calibration curve).

The fluorescence of the eluted fractions was determined as follows:- Firstly, the volume of each eluted fraction was measured visually from the graduated vials (to the nearest 0.1ml). An aliquot of each fraction (1ml) was transferred to a clean sample cup. H33258/buffer solution (see above) was added to each aliquot, mixed and the fluorescence determined as described above. The amount of DNA in each fraction was calculated taking into account the volume of the fraction and the DNA concentration (ug ml^{-1}) as determined from the calibration curve.

The amount of DNA retained on the filter was determined by measuring the fluorescence of an aliquot (1ml) of the filter wash/sonicate as described above. The volume of each filter wash was determined using a measuring cylinder (25ml).

3.2.4.7 *Presentation of results*

Results were plotted semi-logarithmically as the log of the proportion

of DNA remaining of filter against cumulative volume eluted (ml). From this graph the Strand Scission Factor (SSF) can be derived using the following equation:-

$$SSF = -\log (F_x/F_c)$$

Where F_x is the fraction of DNA retained on the filter of the treated sample at the midpoint volume of the total volume eluted, and F_c is the corresponding value for the control sample. SSF is used to relate drug induced DNA strand scission to that caused by radiation in the same system. Drug induced strand scission can therefore be expressed as radiation (Rad or Gray) equivalents. This enables direct comparison of the DNA strand scission caused by different drugs in either the same system or different systems.

3.3 Results

3.3.1 *Effect of quinone antitumour agents on plasmid DNA in the presence of purified reductase enzymes.*

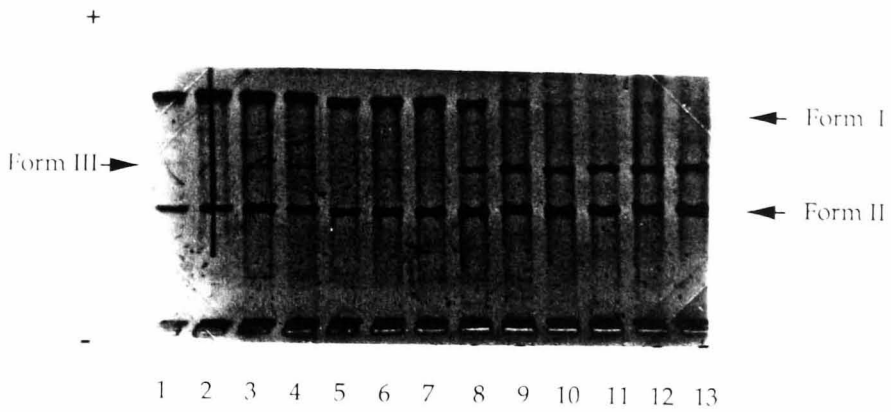
3.3.1.1 *Effect of doxorubicin on strand breakage of plasmid DNA in the presence of xanthine oxidase and cytochrome P450 reductase.*

Studies were carried as described in section 3.2.1.

It can be seen from figure 3.1A and photograph 3.1 that doxorubicin, in the presence of xanthine oxidase and NADH, produced strand breakage of plasmid DNA in a concentration dependent manner. As the doxorubicin concentration was increased the proportion of both open circular and linear DNA increased, indicating a dose dependent formation of single and double strand breaks. No significant strand breakage was seen in the absence of drug or when NADH was removed from the system [see figure 3.2A], indicating strand breakage was doxorubicin and NADH dependent.

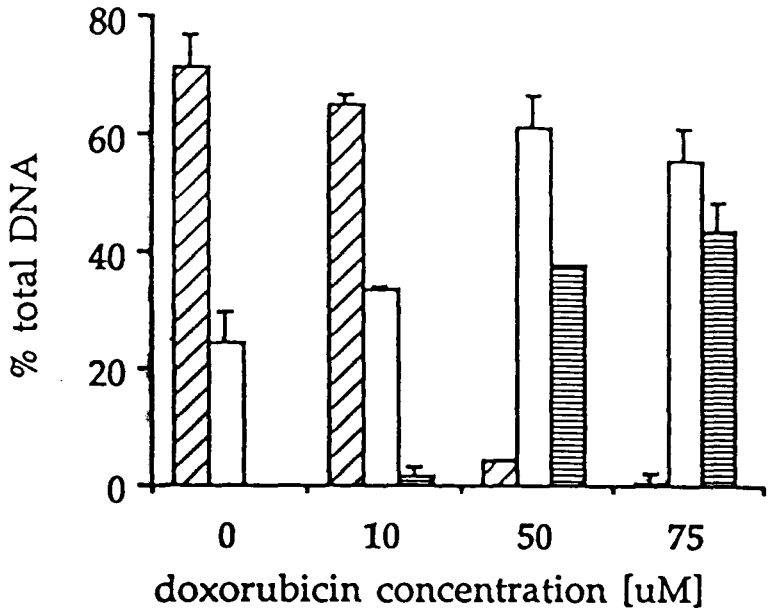
Figure 3.1B shows that doxorubicin produced a concentration dependent increase in strand breakage in the presence of cytochrome P450 reductase and NADPH. However double strand breakage was only seen at the highest drug concentration. Enzyme and NADPH alone caused no significant strand breakage. When NADPH was removed from the system [see figure 3.2B] there was no significant strand breakage by doxorubicin [100uM], indicating this cofactor was required for strand breakage.

For subsequent studies drug concentrations of 50uM [for xanthine oxidase] and 100uM [for cytochrome P450 reductase] were used as doxorubicin at these concentrations produced strand breakage.



Photograph 3.1 *The effect of doxorubicin on plasmid DNA in the presence of xanthine oxidase.*

Plasmid DNA (300ng) [**lanes 1+2**] was incubated (37C, 30min) with xanthine oxidase (2.5units) and NADH (0.4mM) [**lanes 3+4**], and doxorubicin (10μM) [**lanes 5-7**], (50μM) [**lanes 8-10**], and (75μM) [**lanes 11-13**].



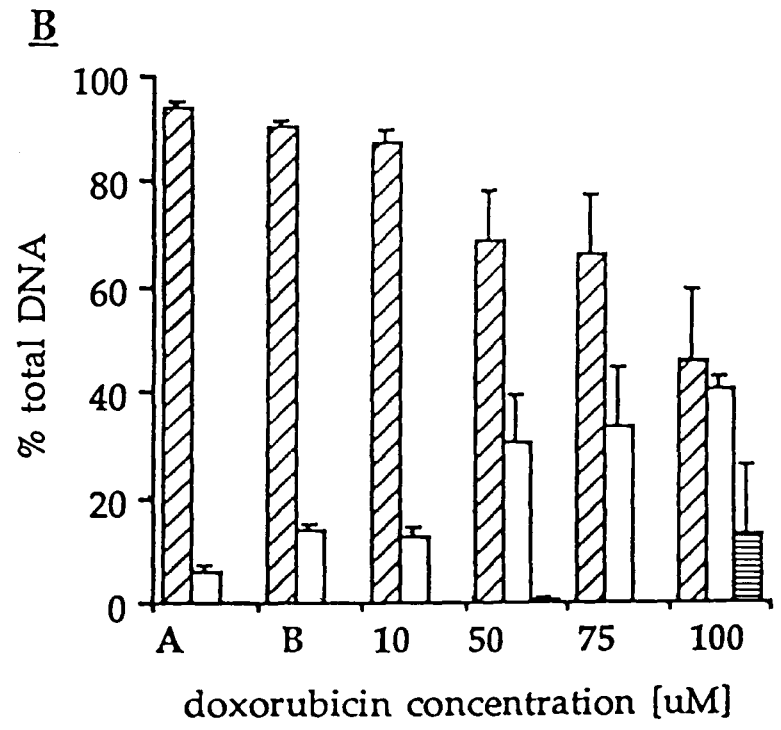


Figure 3.1 *Effect of doxorubicin on strand breakage of plasmid DNA in the presence of xanthine oxidase and cytochrome P450 reductase.*

Doxorubicin was incubated with plasmid DNA [300ng] and A: xanthine oxidase [2.5 units] and NADH [4.0mM] or B: cytochrome P450 reductase [23.4 units] and NADPH [1.0mM] for 30 minutes at 37C [controls were DNA (A), DNA + cytochrome P450 reductase (B)]. DNA species resolved by gel electrophoresis were supercoiled [▨], open circular [□] and linear [▤]. Values are the mean + sd of three determinations.

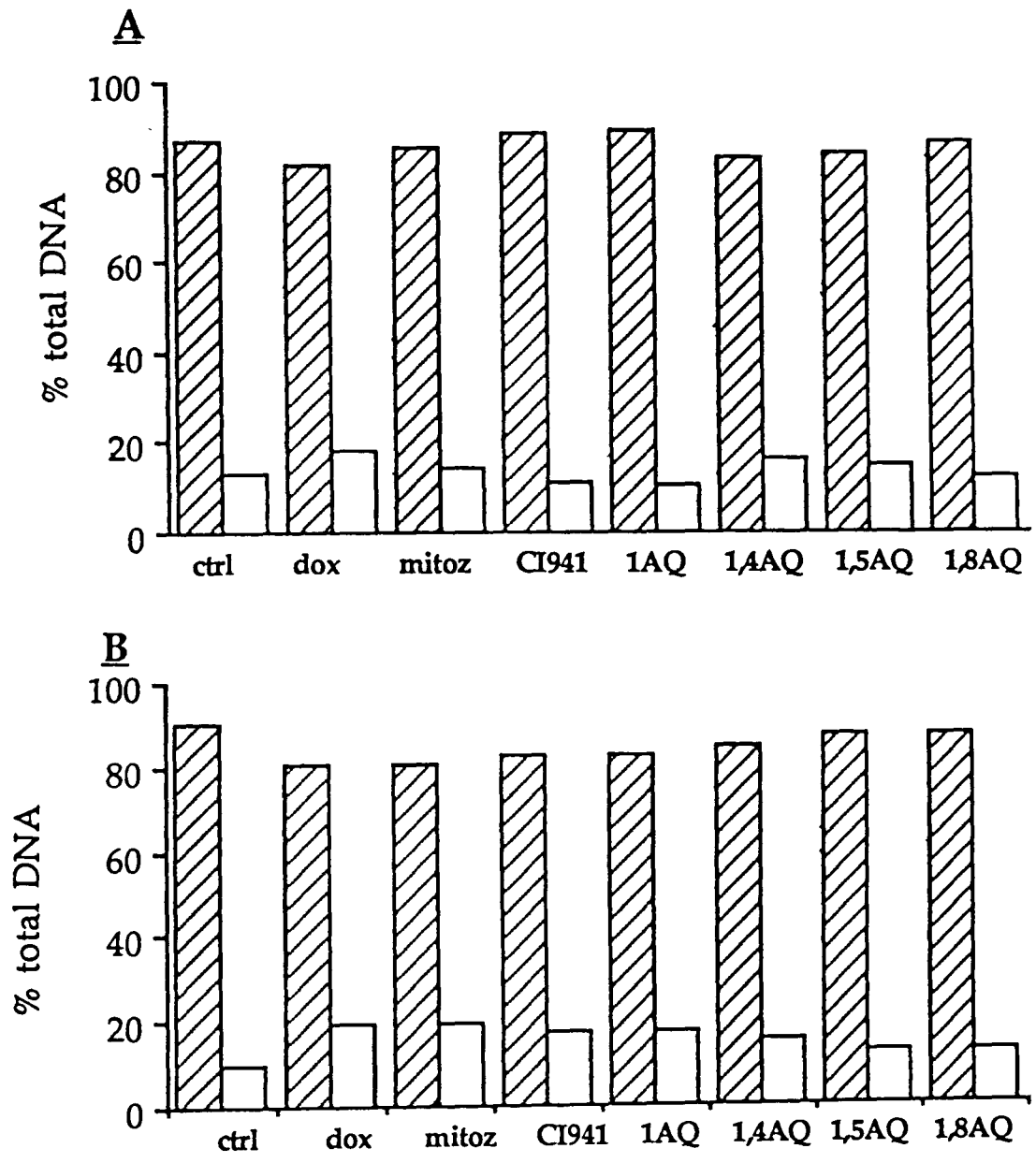


Figure 3.2 *Effect of doxorubicin, mitozantrone, CI941 and alkylamino-anthraquinones on strand breakage of plasmid DNA in the presence of xanthine oxidase [A] and cytochrome P450 reductase [B].*

Incubations were carried out as described in figure 3.1 but in the absence of NADH or NADPH. Control incubation consisted of DNA only [Ctrl]. DNA species resolved by gel electrophoresis were supercoiled [▨] and open circular [□].

3.3.1.2 *Effect of mitozantrone and CI941 on plasmid DNA in the presence of xanthine oxidase and cytochrome P450 reductase*

Figure 3.3A shows that CI941 [50uM] had no significant effect on plasmid DNA strand breakage in the presence of xanthine oxidase and NADH. Mitozantrone [50uM] produced a small increase in open circular DNA relative to controls [enzyme and NADH only]. This indicated some single strand breakage by mitozantrone. Figure 3.3A shows that in the absence of NADH, mitozantrone produced no significant increase in open circular DNA relative to control.

Figure 3.2B and 3.3B show that CI941 produced a similar level of plasmid DNA strand breakage both in the presence and absence of NADPH in a system containing cytochrome P450 reductase suggesting strand breakage was not enzyme cofactor dependent. Mitozantrone, in this system [figure 3.3B] produced single and double strand breakage which was concentration dependent. At 100uM mitozantrone all the plasmid DNA contained single or double strand breaks. In the absence of mitozantrone or NADPH (figure 3.2B) no strand breakage was observed in this system.

3.3.1.3 *Effect of alkylaminoanthraquinones on plasmid DNA in the presence of xanthine oxidase and cytochrome P450 reductase*

No significant strand breakage was detected for the AQ's in the presence of xanthine oxidase and NADH [figure 3.5A].

Figure 3.5B shows that 1AQ [4.4 fold], 1,5AQ [5.3 fold] and 1,8AQ [4.8 fold] at 100uM all produced a large increase in the amount of open circular DNA in a system containing cytochrome P450 reductase and NADPH. Cytochrome P450 reductase and NADPH alone produced no significant increase in strand breakage. In the absence of NADPH [figure 3.2B] 1AQ, 1,5AQ and 1,8AQ (100uM) produced only a slight increase in open circular

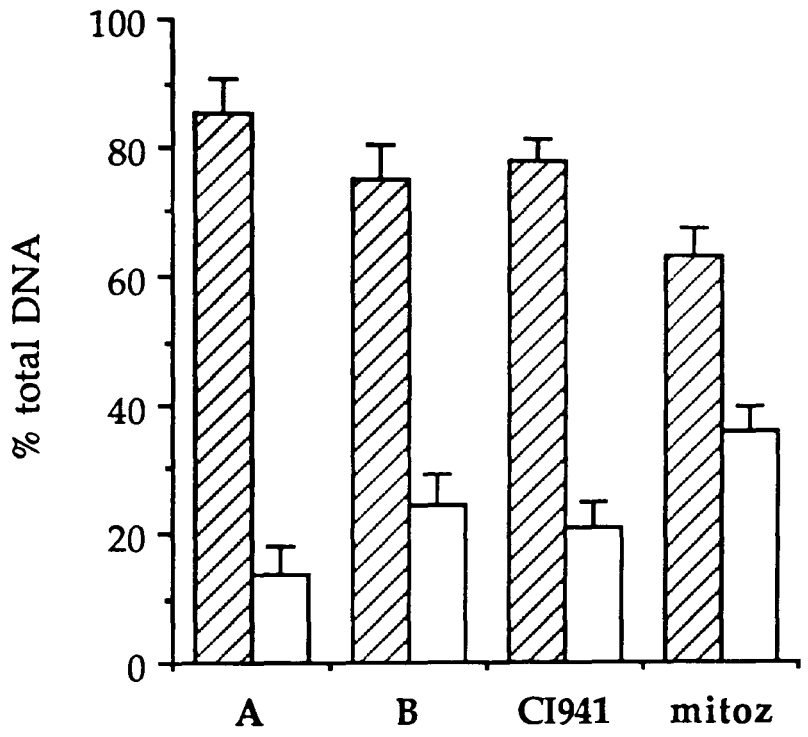


Figure 3.3 *Effect of mitozantrone and CI941 on strand breakage of plasmid DNA in the presence of xanthine oxidase*

Drugs [50uM] were incubated [37C, 30min] with plasmid DNA [300ng], xanthine oxidase [0.25 units] and NADH [4.0mM]. Control A:- DNA only, Control B:- DNA, xanthine oxidase and NADH. DNA species resolved by gel electrophoresis were supercoiled [▨], open circular [□] and linear. Values are the mean + sd of three determinations.

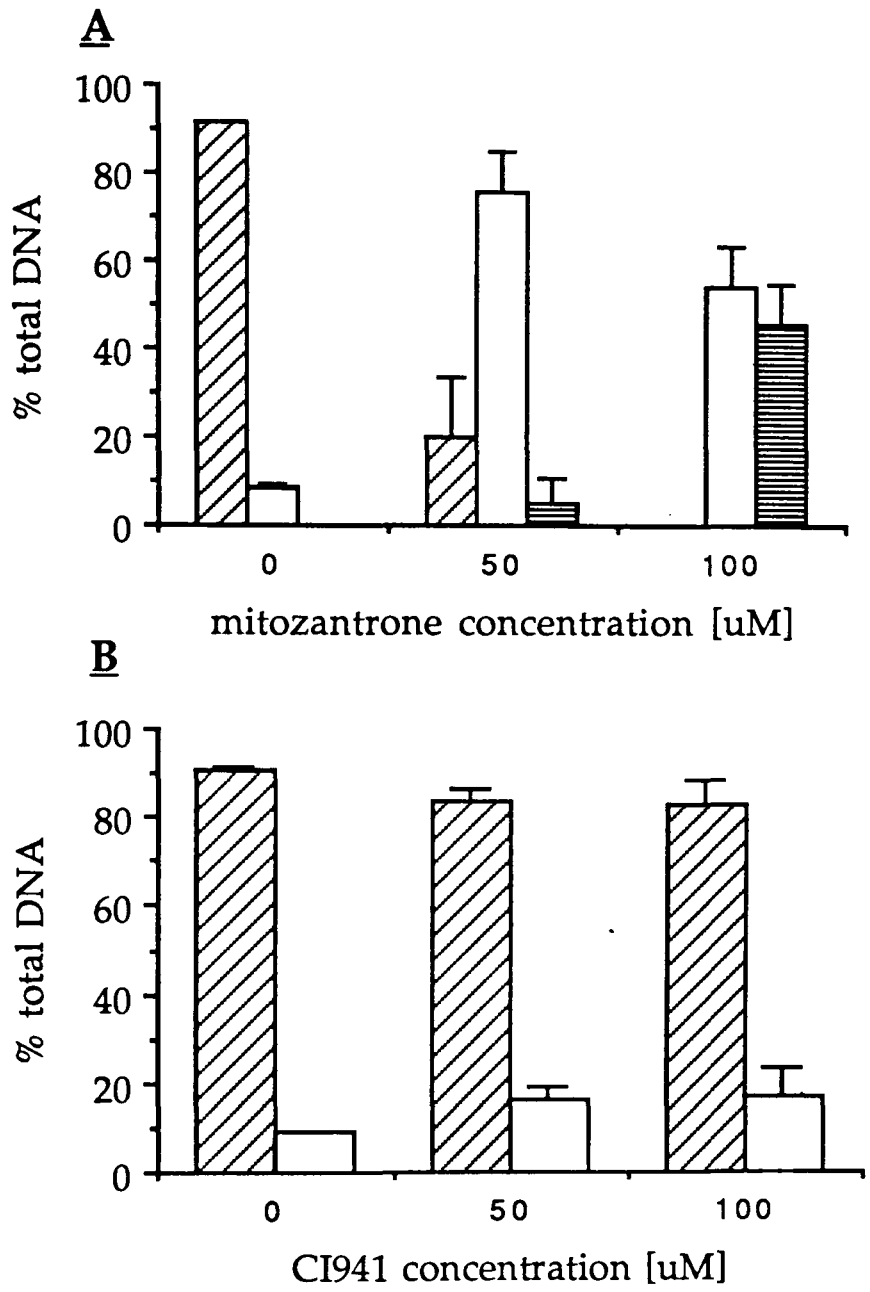


Figure 3.4 *Effect of mitozantrone [A] and CI941 [B] on strand breakage of plasmid DNA in the presence of cytochrome P450 reductase.*

Drugs [100uM] were incubated [37C, 30min] with plasmid DNA [300ng], cytochrome P450 reductase [23.4 units] and NADPH [1.0mM]. DNA species resolved by gel electrophoresis were supercoiled [▨], open circular [□] and linear [▤]. Values are the mean + sd of three determinations.

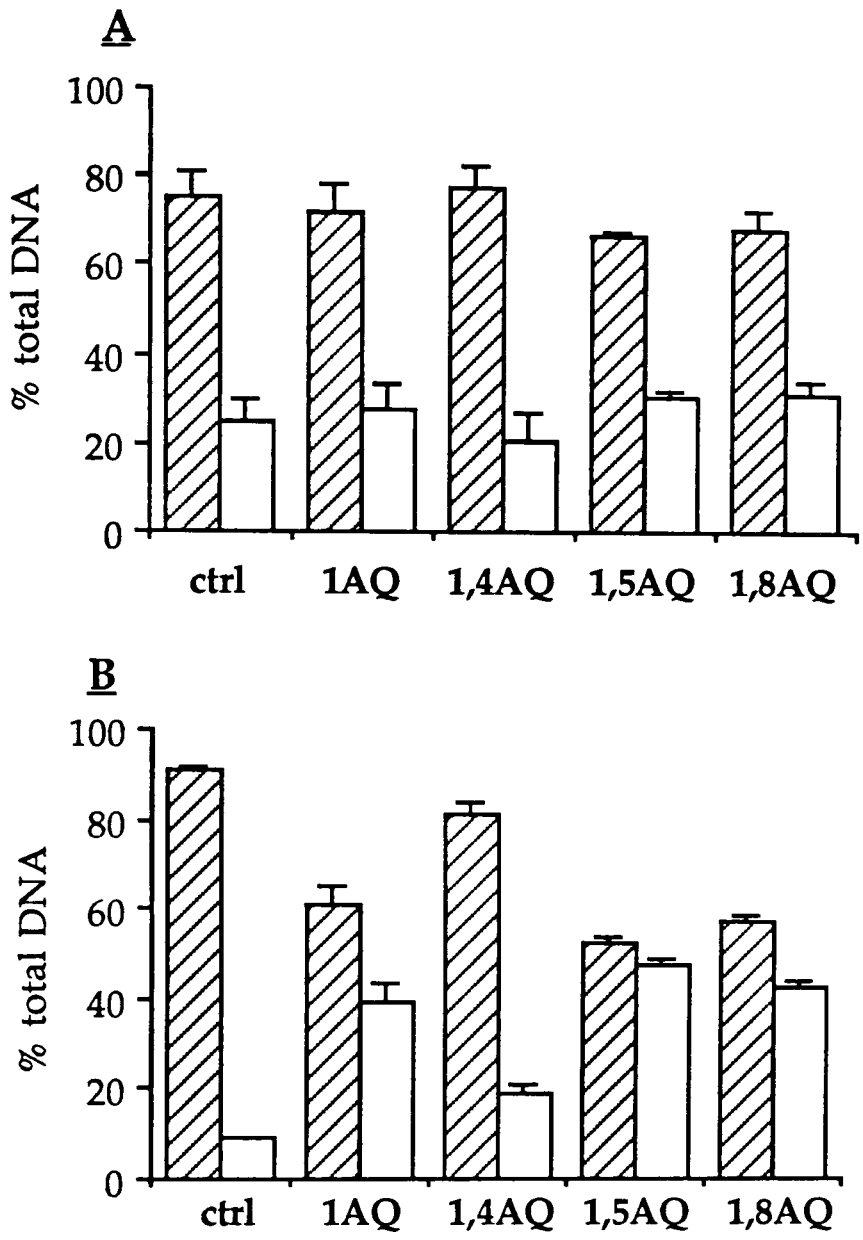


Figure 3.5 *Effect of alkylaminoanthraquinones on strand breakage of plasmid DNA in the presence of xanthine oxidase and cytochrome P450 reductase.*

A: AQ's [50uM] were incubated with xanthine oxidase as described in figure figure 3.4,**B:** AQ's [100uM] were incubated with cytochrome P450 reductase as described in figure 3.5. Control consisted of DNA + enzyme and NAD(P)H. DNA species resolved by gel electrophoresis were supercoiled [▨] and open circular [□]. Values are the mean + sd of at least three determinations.

DNA relative to DNA alone. These results indicated that DNA strand breakage in the presence of cytochrome P450 reductase by these AQ's was drug and NADPH dependent. The 1,4AQ produced no significant strand breakage in this system.

3.3.2 *Effect of quinone antitumour agent iron complexes on strand breakage of plasmid DNA in the presence of glutathione.*

3.3.2.1 *The effect of doxorubicin and mitozantrone iron complexes on strand breakage of plasmid DNA in the presence of reduced glutathione.*

A doxorubicin-iron complex was formed using ferrous ammonium sulphate (1:2 drug:iron ratio) as described in section 3.2.2.1. The effect of this complex on plasmid DNA in the presence of reduced glutathione is shown in figure 3.6. The relative intensities of DNA species in the presence of glutathione only were supercoiled 69.4 + 14.1 and open circular 14.75 + 3.1. In the presence of ferrous iron (100uM) and glutathione the intensities of supercoiled and open circular DNA were typically 51.8 and 74.0 respectively. Doxorubicin-iron complex produced a concentration dependent decrease in the total amount of supercoiled and open circular DNA due to frank double strand breakage. The complex at 5:10 and 25:50 uM (drug:iron) produced some double strand breaks and at 50:100 uM destroyed all the DNA present. This did not occur in the presence of drug only.

A mitozantrone-iron complex was prepared using ferrous ammonium sulphate at a 1:2 drug:iron ratio. Photograph 3.2 shows the result when the effect of this complex on plasmid DNA in the presence of reduced glutathione was investigated. It can be seen that mitozantrone-iron complex 50:100uM (drug:iron) produced double strand breakage of the DNA. A blue precipitate was observed following the DNA ethanol precipitation step despite carrying out five n-butanol extractions. The DNA treated

with complex was seen to 'streak' rather than run in discrete bands as seen in the other lanes. This suggested that the mitozantrone-iron complex did not extract from the DNA as efficiently as drug alone. The lanes of this photograph were quantitated and are shown in figure 3.7. This clearly shows that mitozantrone-iron complex caused double strand breaks in this system whilst iron or drug alone at equivalent concentrations produced no such effect.

3.3.2.2 *The effect of CI941 and related anthrapyrazole iron complexes on strand breakage of plasmid DNA in the presence of reduced glutathione*

A CI941-iron complex was prepared as described in section 3.2.2.1 using ferric chloride with a drug:iron ratio of 1:2. The complex formed showed no significant effect on plasmid DNA in the presence of reduced glutathione relative to controls [figure 3.8].

Ferric chloride was used to prepare anthrapyrazole [APZ] iron complexes for CI941 and related anthrapyrazoles [APZ's] shown in figure 1.36. Figure 3.9 and photograph 3.3 shows that the 7,10-H-APZ iron complex produced no significant DNA strand breakage in the presence of reduced glutathione relative to controls. However the 7,10-OH-APZ and 10-OH-APZ iron complexes produced single and double strand breaks both in the presence and absence of oxygen. In the absence of glutathione these complexes produced no effect on the DNA. Ferric iron and glutathione alone also showed no effect.

3.3.2.3 *The effect of alkylaminoanthraquinones on the strand breakage of plasmid DNA in the presence of iron and reduced glutathione.*

Mixtures of AQ's and ferrous iron (1:2 drug:iron ratio) were prepared as described in section 3.2.2.1 and the effect of these mixtures on plasmid

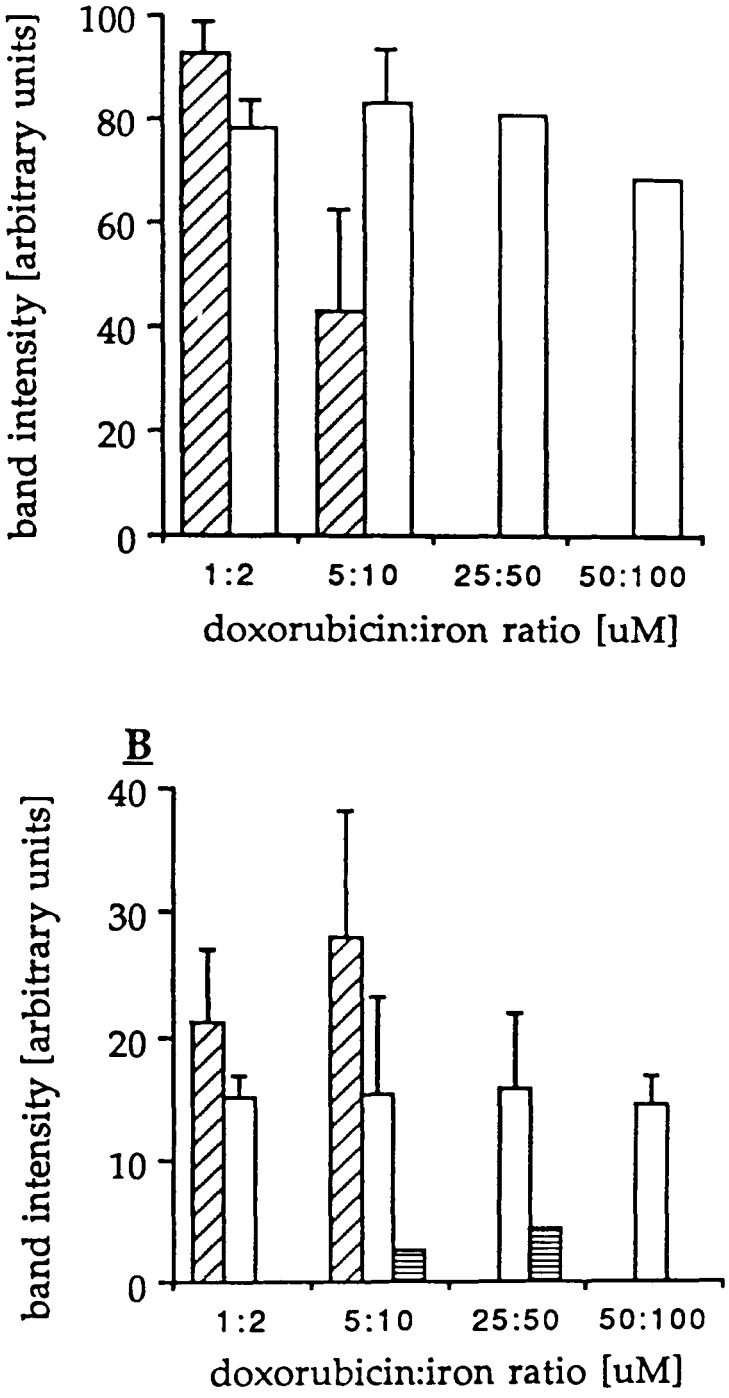
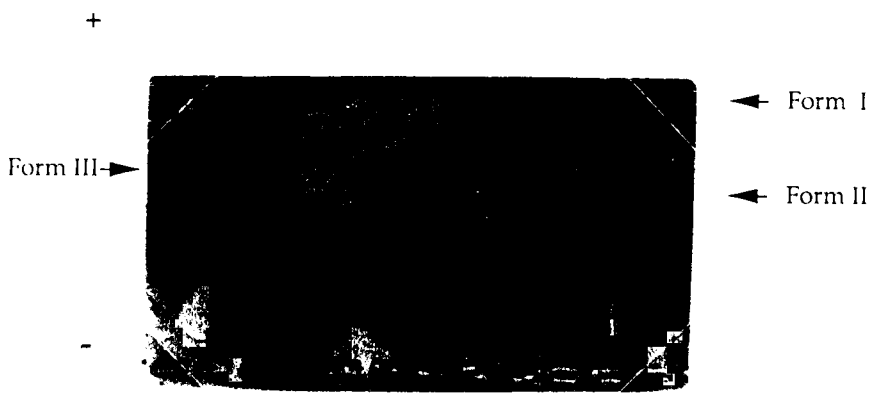


Figure 3.6 *Effect of doxorubicin-iron complex on strand breakage of plasmid DNA in the presence of reduced glutathione.*

Graphs are A: supercoiled DNA and B: open circular DNA.
Plasmid DNA [300ng] was incubated (30min, 37C) with doxorubicin-ferrous iron complex [⊘] or doxorubicin [○]. Values are mean + sd of three determinations. [⊜] - linear DNA produced by doxorubicin-ferrous iron complex.



Photograph 3.2 *The effect of mitozantrone-iron complex on strand breakage of plasmid DNA in the presence of reduced glutathione.*

Plasmid DNA (300ng) was incubated (37C, 15min) with glutathione (2.5μM) [**lanes 1-3**], glutathione and iron(II) (100μM) [**lanes 4-5**], glutathione and mitozantrone (50μM) [**lanes 7-9**] and glutathione and mitozantrone-iron(II) complex (50μM:100μM) [**lanes 10-12**].

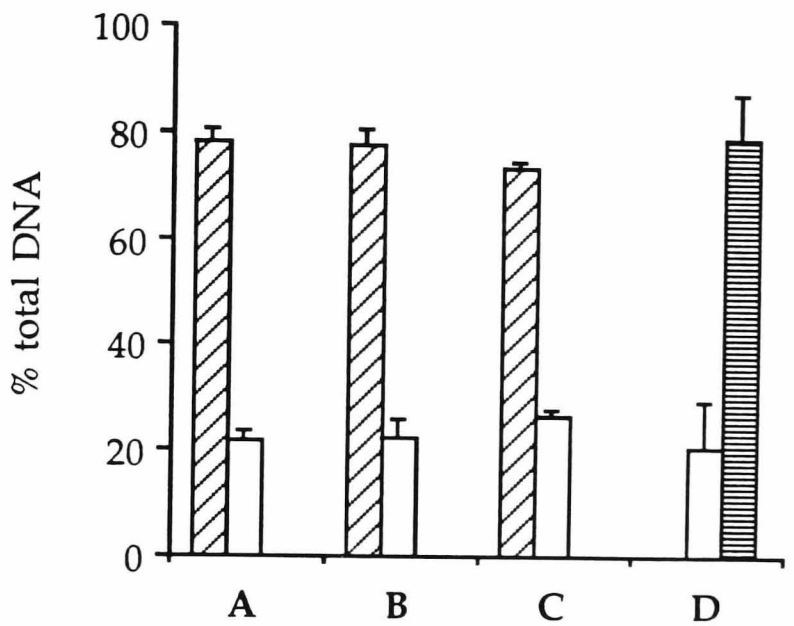


Figure 3.7 *The effect of mitozantrone-iron complex on strand breakage of plasmid DNA in the presence of reduced glutathione.*

Plasmid DNA [300ng] was incubated [37C, 30min] with glutathione (2.5uM) [**A**], glutathione and ferrous iron (100uM) [**B**], glutathione and mitozantrone (50uM) [**C**] and glutathione and mitozantrone-ferrous iron complex (50:100 uM) [**D**]. The DNA species resolved by gel electrophoresis were [▨] supercoiled, [□] open circular and [▤] linear. Values are mean + sd of three determinations. Data derived from photograph 3.1.

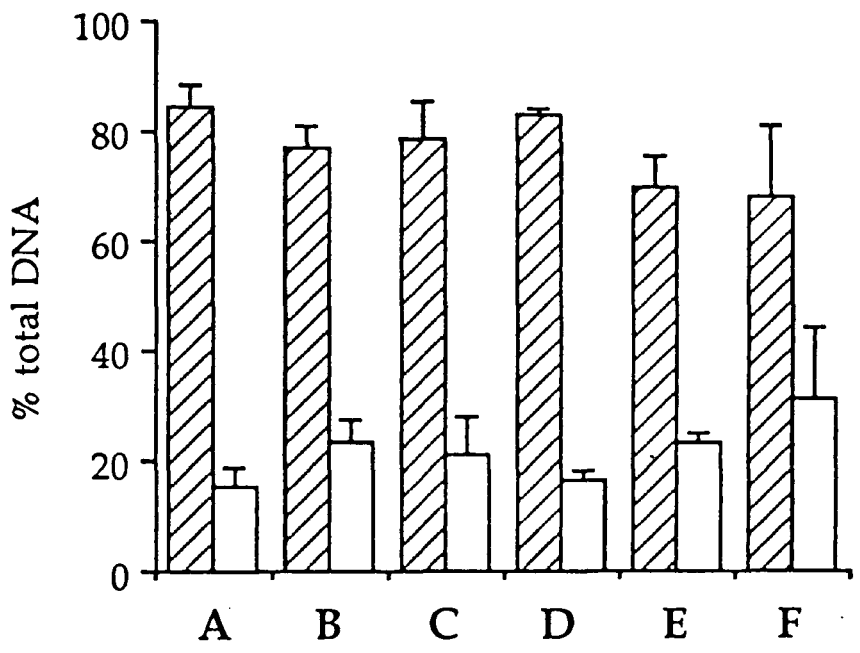
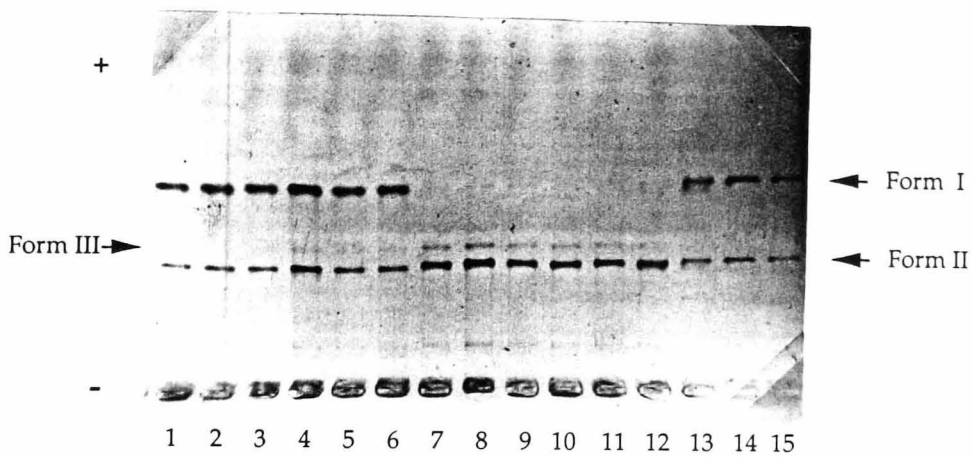


Figure 3.8 *Effect of CI941-iron complex on strand breakage of plasmid DNA in the presence of reduced glutathione.*

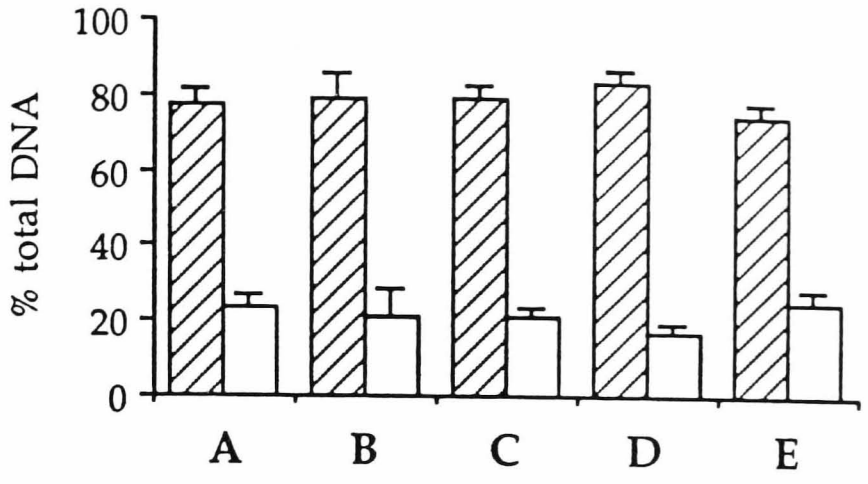
Plasmid DNA (300ng) [A] was incubated (30min, 37C) with glutathione (2.5mM) [B], glutathione and ferric iron (100uM) [C], CI941-ferric iron complex (50:100uM) [D], glutathione and CI941-ferric iron complex (50:100uM) [E], and glutathione and CI941-ferric iron complex (50:100uM) deaerated with nitrogen [F]. The DNA species resolved by gel electrophoresis were [⊘] supercoiled and [○] open circular. Values are the mean + sd of three determinations.



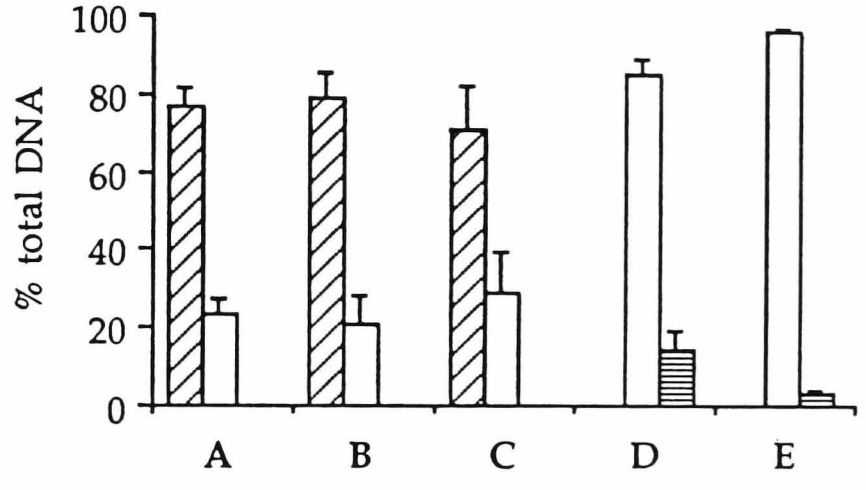
Photograph 3.3 *Effect of APZ-iron complexes on strand breakage of plasmid DNA in the presence of reduced glutathione*

Plasmid DNA (300ng) was incubated with glutathione (2.5mM) and Fe(III) (100μM) [**lanes 1-3**], 7,10-H APZ+Fe(III) (50:100μM) [**lanes 4-6**], 10-OH APZ + Fe(III) (50:100μM) [**lanes 7-9**], 7,10-OH APZ + Fe(III) (50:100μM) [**lanes 10-12**] and 7-OH APZ + Fe(III) (50:100μM) [**lanes 13-15**].

_ _: 7,10-H



B: 7,10-OH



C: 10-OH

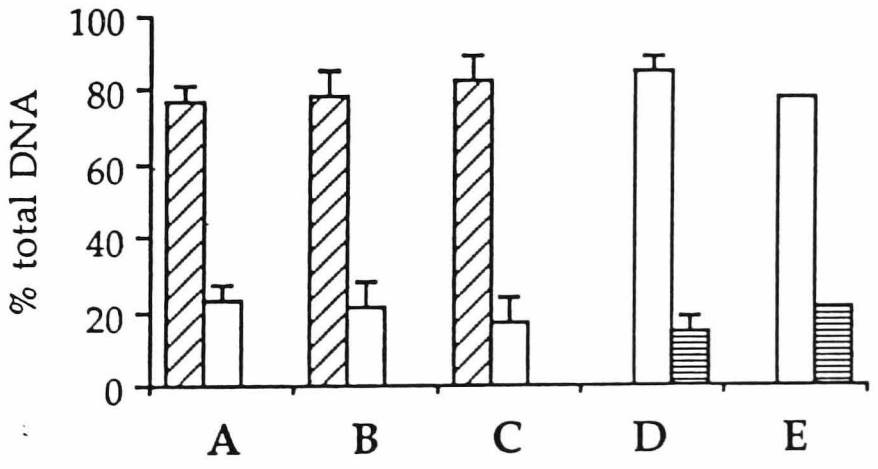


Figure 3.9 *Effect of anthrapyrazole-iron complexes on strand breakage of plasmid DNA in the presence of reduced glutathione.*

Plasmid DNA (300ng) was incubated with glutathione (2.5mM) [A], glutathione and ferric iron (100uM) [B], complex (50:100 uM ferric iron:drug) [C], glutathione and complex [D] and glutathione and complex deaerated with nitrogen [E]. The DNA species resolved by gel electrophoresis were [⊘] supercoiled, [○] open circular and [⊖] linear. Values are mean + sd of three determinations.

DNA in the presence of reduced glutathione determined. Figure 3.10 shows that in the presence of ferrous iron and glutathione the AQ's produced no significant DNA strand breakage relative to controls containing glutathione and drug only.

3.3.3 *Effect of quinone antitumour agents on topoisomerase activity in MCF-7 nuclear extract.*

A nuclear extract was isolated from MCF-7 cells as described in section 3.2.3.1. Supercoiled plasmid DNA is a substrate for topoisomerase I and II activity resulting in the relaxation of the DNA to the open circular form. These two types of enzyme can be discriminated by the requirement of ATP for topoisomerase II activity. It can be seen from photograph 3.4 that in the presence of MCF-7 nuclear extract pBR322 supercoiled DNA was converted to open circular, relaxed DNA and a further series of bands of intermediate mobility between open circular and supercoiled DNA. These bands are the result of different degrees of unwinding of the supercoiled DNA molecule and are known as concatomers or topomers (reviewed by Maxwell and Gellert, 1986), being characteristically produced by topoisomerase activity [section 1.7]. There was no difference in DNA unwinding in the presence or absence of ATP. It can also be seen that the intensity of the topomer banding increases with increasing extract protein concentration. Doxorubicin (1uM) inhibited the unwinding or relaxing activity produced at all concentrations of nuclear extract used. From this experiment an extract concentration of 1.0ug protein was chosen for subsequent studies.

In order to identify the type of topoisomerase responsible for the DNA unwinding activity present in MCF-7 nuclear fraction the effect of the topoisomerase II inhibitors mAMSA (Nelson *et al.*, 1984) and amiloride (Besterman *et al.*, 1987) and the topoisomerase I inhibitor camptothecin (Hsiang and Liu, 1988) [figure 3.18] on this activity was investigated. The reaction was stopped with either SDS or SDS and proteinase K as described in section 3.2.3. Photograph 3.5 shows the result of this study. It can be seen that none of these drugs at

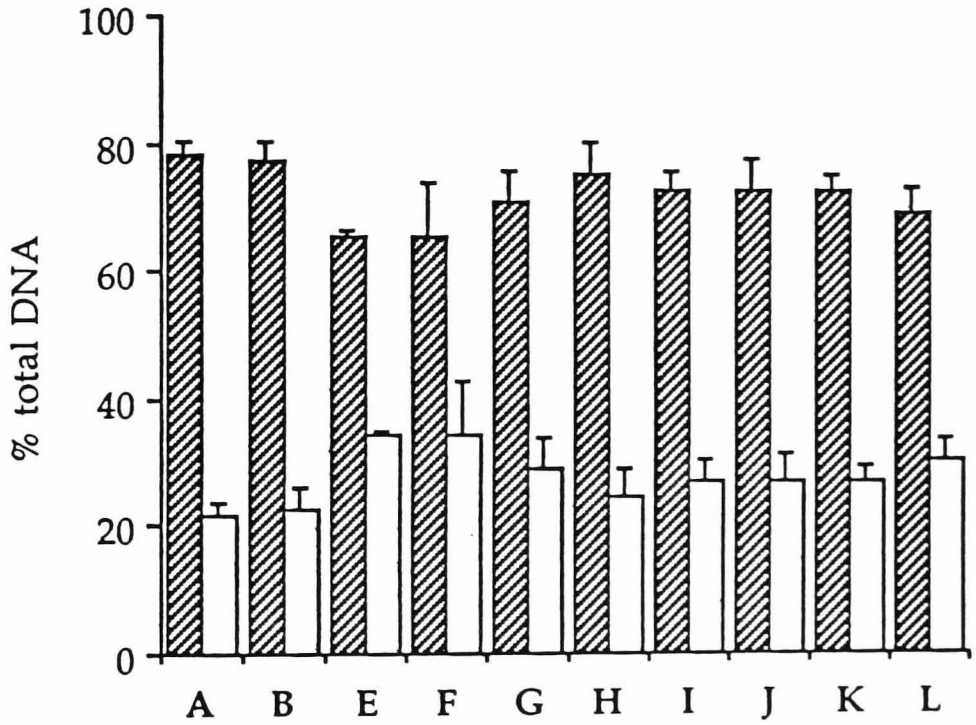


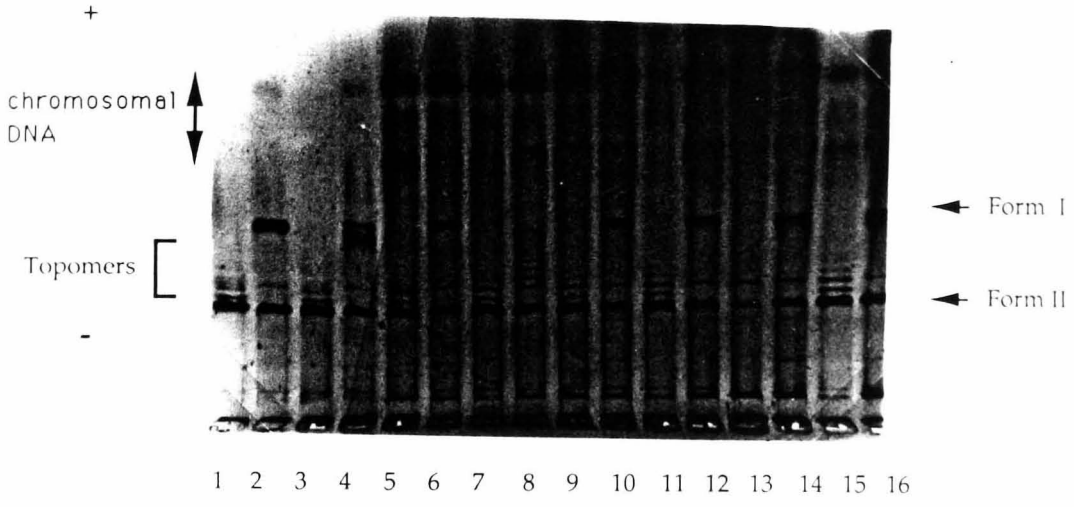
Figure 3.10 *Effect of alkylaminoanthraquinones on strand breakage of plasmid DNA in the presence of ferrous iron and glutathione.*

A drug:ferrous iron concentration of 50:100uM was used. Plasmid DNA (300ng) was incubated (37C, 30min) with glutathione (2.5mM) [**A**], glutathione and iron (100uM) [**B**] or glutathione and:- 1AQ [**E**], 1AQ and iron [**F**], 1,4AQ [**G**], 1,4AQ and iron [**H**], 1,5AQ [**I**], 1,5AQ and iron [**J**], 1,8AQ [**K**] and 1,8AQ and iron [**L**]. DNA species resolved by gel electrophoresis were supercoiled [▨] and open circular [□]. Values are mean + sd of three determinations.

100ng/ml inhibited DNA unwinding activity. Formation of topoisomerase cleavable complexes is revealed by digestion with proteinase K which results in formation of single and double strand breaks hence an increase in open circular and linear DNA [see section 1.7]. However, no difference in the DNA banding (ie amount of topomers)[photograph 3.5B] was seen when the reaction was stopped with SDS and proteinase k. The effect of doxorubicin and a higher concentration of mAMSA on DNA unwinding activity of MCF-7 nuclear extract was further investigated (photograph 3.6). It was found that when the reaction was stopped with SDS and proteinase K both doxorubicin (0.1uM) and mAMSA (10uM) showed a slight inhibition of topomer formation in the presence and absence of ATP. Doxorubicin inhibited topomer formation to an equal extent when the reaction was stopped by either method. Subsequently the effect of doxorubicin concentration on the DNA unwinding activity of MCF-7 nuclear extract in the presence of ATP was determined. Photograph 3.7 shows that the inhibition of DNA unwinding activity by doxorubicin was concentration dependent as indicated by the reduction of topomer formation with increasing doxorubicin concentration. Doxorubicin (5uM) totally inhibited DNA unwinding activity. When the reaction was stopped with SDS and proteinase K [as described in section 3.2.3] no increase in open circular or linear DNA was produced.

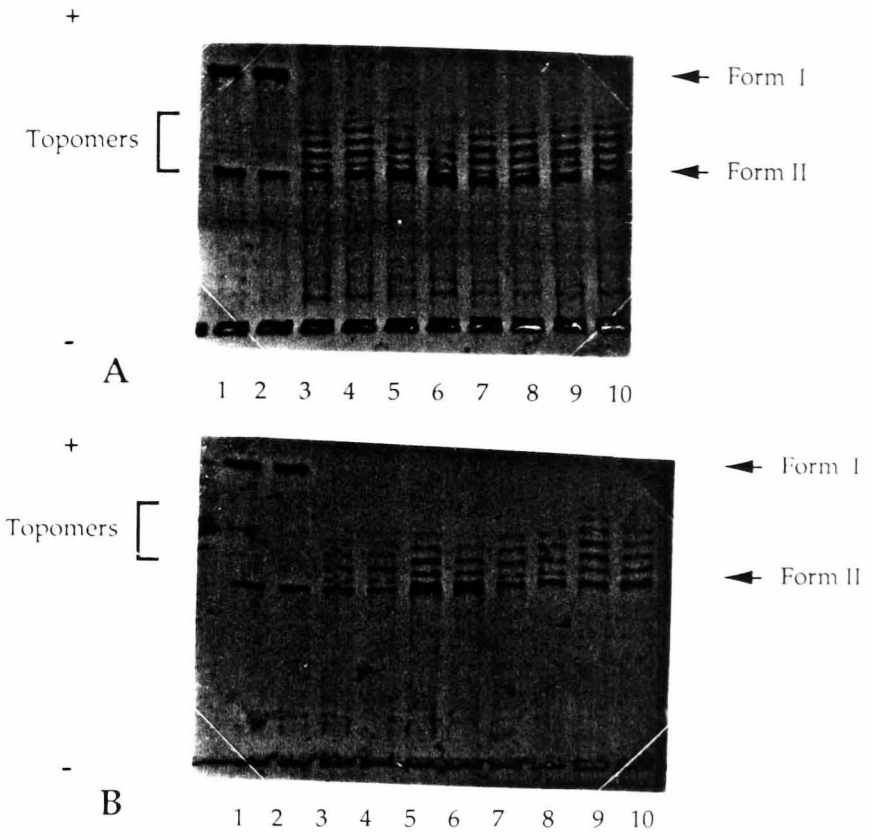
A drug concentration of 1uM was chosen to investigate the effect of the other quinone antitumour agents used in this study on the DNA unwinding activity of MCF-7 nuclear extract (Photograph 3.8). When the reaction was stopped with SDS only, mitozantrone and CI941 appeared to be the most potent inhibitors of this activity as they totally inhibited unwinding of supercoiled DNA. 1,4AQ, 1,5AQ and 1,8AQ produced a similar level of inhibition, but less than mitozantrone or CI941. 1AQ showed the least inhibition of unwinding activity. When the reaction was stopped with SDS and proteinase K no difference in the DNA banding pattern was observed.

3.3.4 The effect of quinone antitumour agents on cellular DNA.



Photograph 3.4 *Topoisomerase activity in MCF-7 cell nuclear extract.*

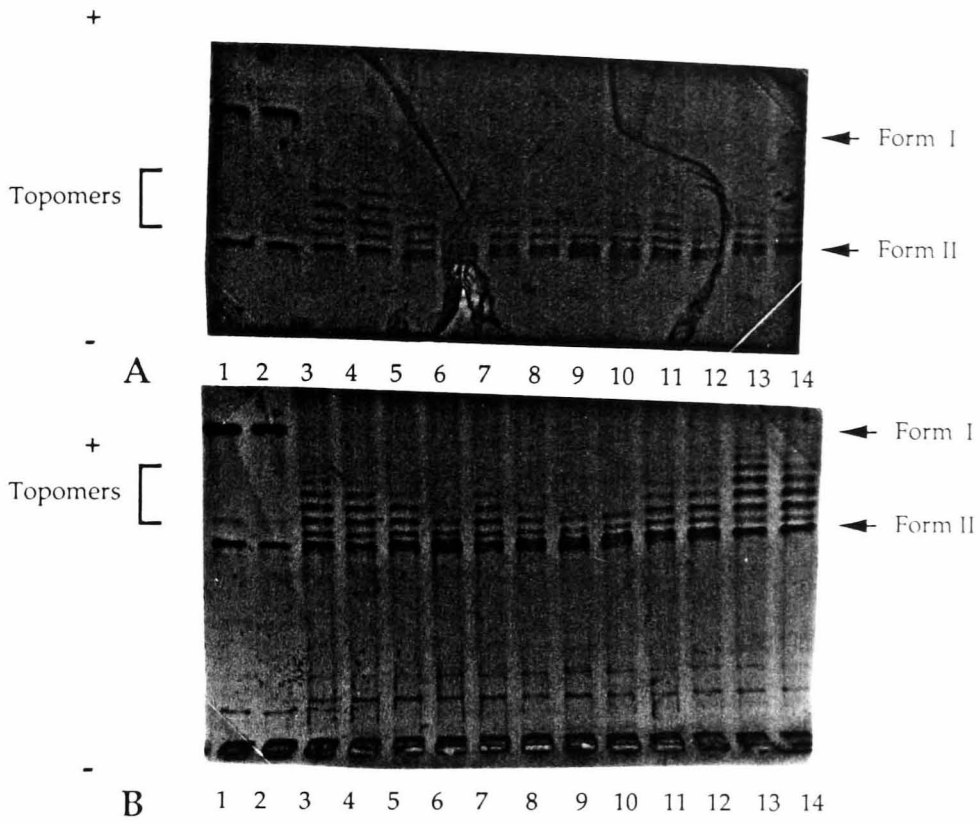
Plasmid DNA (500ng) was incubated (15min) with extract (1µg protein + ATP (1mM) [**lane 1**], +ATP + doxorubicin (1µM) [**lane 2**], -ATP [**lane 3**], -ATP + doxorubicin [**lane 4**]; extract (5.0µg protein) + ATP [**lane 5**], + ATP +doxorubicin [**lane 6**], -ATP [lane 7], -ATP +doxorubicin [**lane 8**]; extract (10.0µg protein) + ATP [**lane 9**], +ATP +doxorubicin [**lane 10**], -ATP [**lane 11**], -ATP +doxorubicin [**lane 12**]; extract (20.0µg protein) + ATP [**lane13**], +ATP +doxorubicin [**lane 14**], -ATP [**lane 15**], -ATP +doxorubicin [**lane 16**]. The reaction was stopped with SDS (2%).



Photograph 3.5 *The effect of amiloride, camptothecin and mAMSA on topoisomerase activity in MCF-7 cell nuclear extract.*

Plasmid DNA (500ng) was incubated (15min) with ATP (1mM) **[lanes 1+2]**, ATP+ extract (1µg protein) **[lanes 3+4]** , ATP + extract+ amiloride (100ng/ml) **[lanes 5+6]**, ATP + extract + camptothecin (100ng/ml) **[lanes 7+8]**, extract + ATP+ mAMSA (100ng/ml **[lanes 9+10]**,

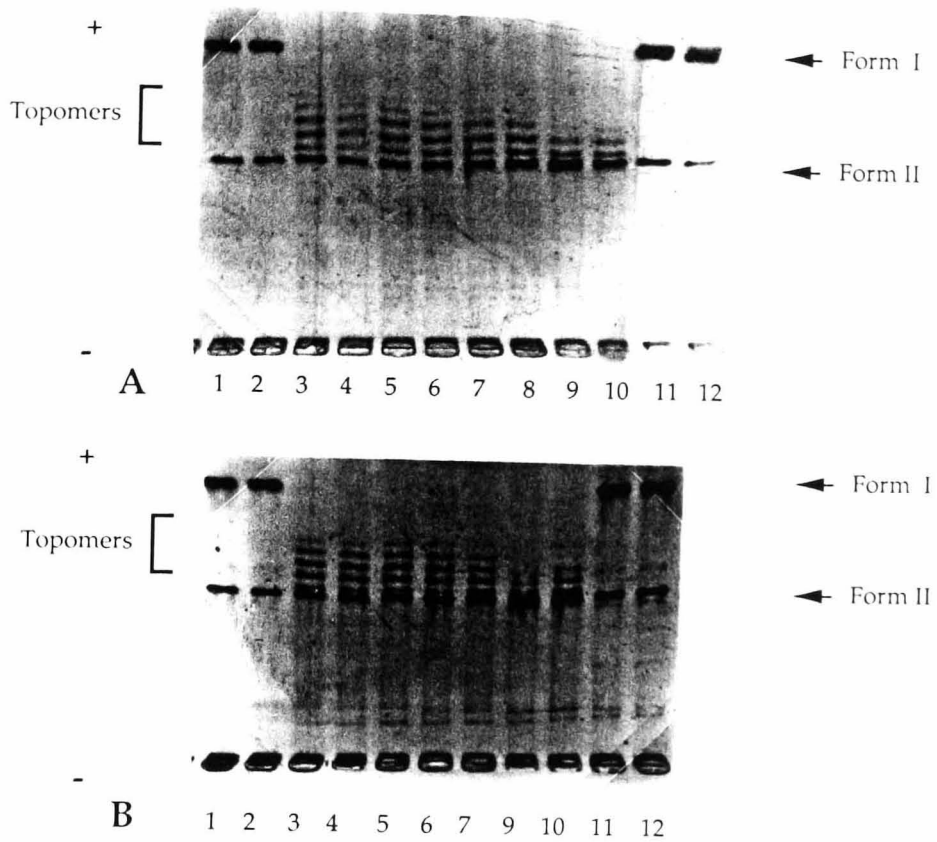
Photograph **A**, reaction stopped with SDS (2%); photograph **B**, reaction stopped with SDS (2%) and proteinase K (0.05mg).



Photograph 3.6 *The effect of doxorubicin and mAMSA on topoisomerase activity in MCF-7 cell nuclear fraction.*

Plasmid DNA (500ng) [**lanes 1+2**] was incubated (15min) with extract (2µg protein) [**lanes 3+4**] or extract+ATP (1mM) [**lanes 5+6**], extract + doxorubicin (0.1µM) [**lanes 7+8**], extract + doxorubicin + ATP [**lanes 9+10**], extract + mAMSA (100µg/ml) [**lanes 11+12**] and extract + mAMSA + ATP [**lanes 13+14**].

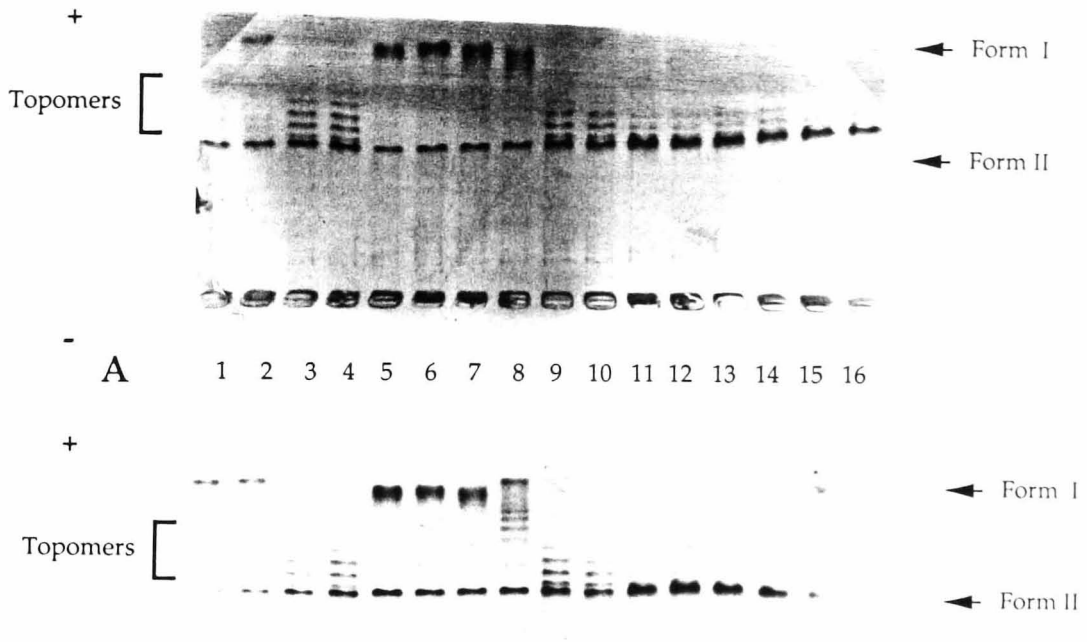
Photograph **A**, reaction stopped with SDS (2%); photograph **B**, reaction stopped with SDS (2%) and proteinase K (0.05mg).



Photograph 3.7 *The effect of doxorubicin on topoisomerase activity in MCF-7 cell nuclear extract.*

Plasmid DNA (500ng) was incubated (15min) with ATP (1mM) **[lanes 1+2]**, ATP+extract (1µg protein) **[lanes 3+4]** or ATP+extract+doxorubicin (0.01µM) **[lanes 5+6]**, (0.05µM) **[lanes 7+8]**, (0.1µM) **[lanes 9+10]** and (5µM) **[lanes 11+12]**.

Photograph **A**, reaction stopped with SDS (2%); photograph **B**, reaction stopped with SDS (2%) and proteinase K (0.1mg).



Photograph 3.8 *The effect of mitozantrone, CI941 and alkylaminoanthraquinones on topoisomerase activity in MCF-7 cell nuclear extract.*

Plasmid DNA (500ng) was incubated (15min) with ATP [**lanes 1+2**], ATP + nuclear extract (1µg protein) [**lanes 3+4**], or ATP + extract + mitozantrone (1µM) [**lanes 5+6**], CI941 (1µM) [**lanes 7+8**], 1AQ (1µM) [**lanes 9+10**], 1,4AQ (1µM) [**lanes 11+12**], 1,5AQ (1µM) [**lanes 13 +14**], and 1,8AQ (1µM) [**lanes 15+16**].

Photograph A reaction stopped with SDS (2%), photograph B reaction stopped with SDS (2%) and proteinase K (0.1mg).

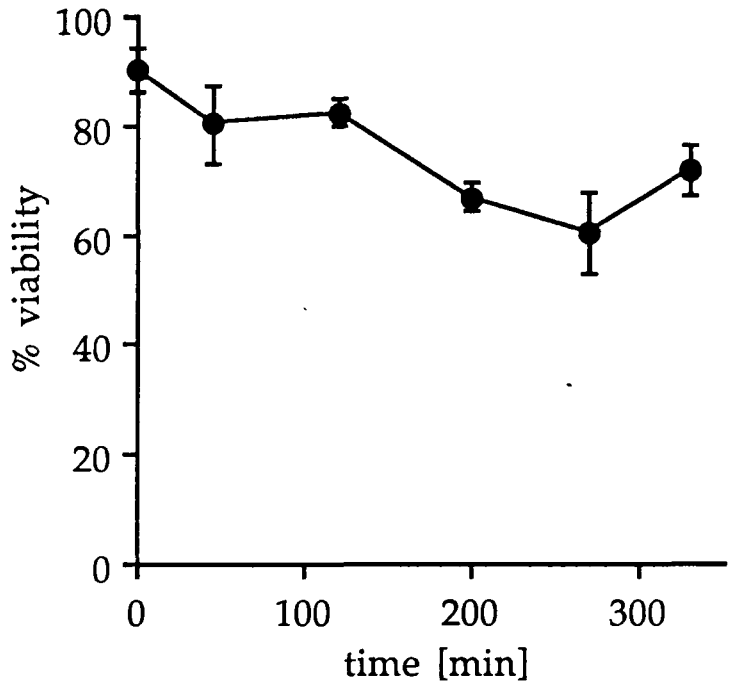
Alkaline [filter] elution studies were carried out in order to determine total strand breakage of DNA caused by quinone antitumour agents in MCF-7 cells. A one hour exposure was chosen to enable comparison with a series of cytotoxicity experiments described in section 4.3.1, in which MCF-7 cells [10^4] were exposed to drug for one hour then washed and incubated for a further six days before assessing cell survival rates. In addition a one hour exposure time has been widely used by other workers to determine cytotoxicity (see tables 4.7 and 4.8) and DNA strand breakage (see section 1.16.4.3). Figure 3.11 shows that the viability of MCF-7 cells in suspension does not decrease significantly over a one hour period even in the presence of drug. The alkaline elution technique is described in section 3.2.4.

3.3.4.1 *The effect of doxorubicin on strand breakage of cellular DNA*

Figure 3.12A shows the elution profile of DNA isolated from MCF-7 cells treated with doxorubicin. It can be seen that as the doxorubicin concentration increased the rate of DNA elution increased relative to untreated controls indicating increasing DNA strand breakage. Figure 3.12B shows the DNA strand scission factors derived from the elution curves in figure 3.12A. These values were derived from the relative proportions of DNA remaining on the filter after half the total elution volume [8ml] had been eluted [see section 3.2.4.7]. This volume was used to derive strand scission factors in all subsequent studies.

3.3.4.2 *Effect of mitozantrone and CI941 on cellular DNA*

Figure 3.13A shows the elution profile of DNA isolated from MCF-7 cells which had been treated with CI941 [100uM] or mitozantrone [100uM] for one hour. There was no difference in the elution rate for either drug, indicating a similar level of strand breakage. Subsequently, strand breakage by these drugs at equitoxic concentrations (ie LD50 values) was investigated. Figure 3.13B shows the elution profile of DNA from MCF-7



3.11 *Survival of harvested MCF-7 cells in suspension.*

Viability of MCF-7 cells was determined using the trypan blue exclusion assay (see appendix A1.9).

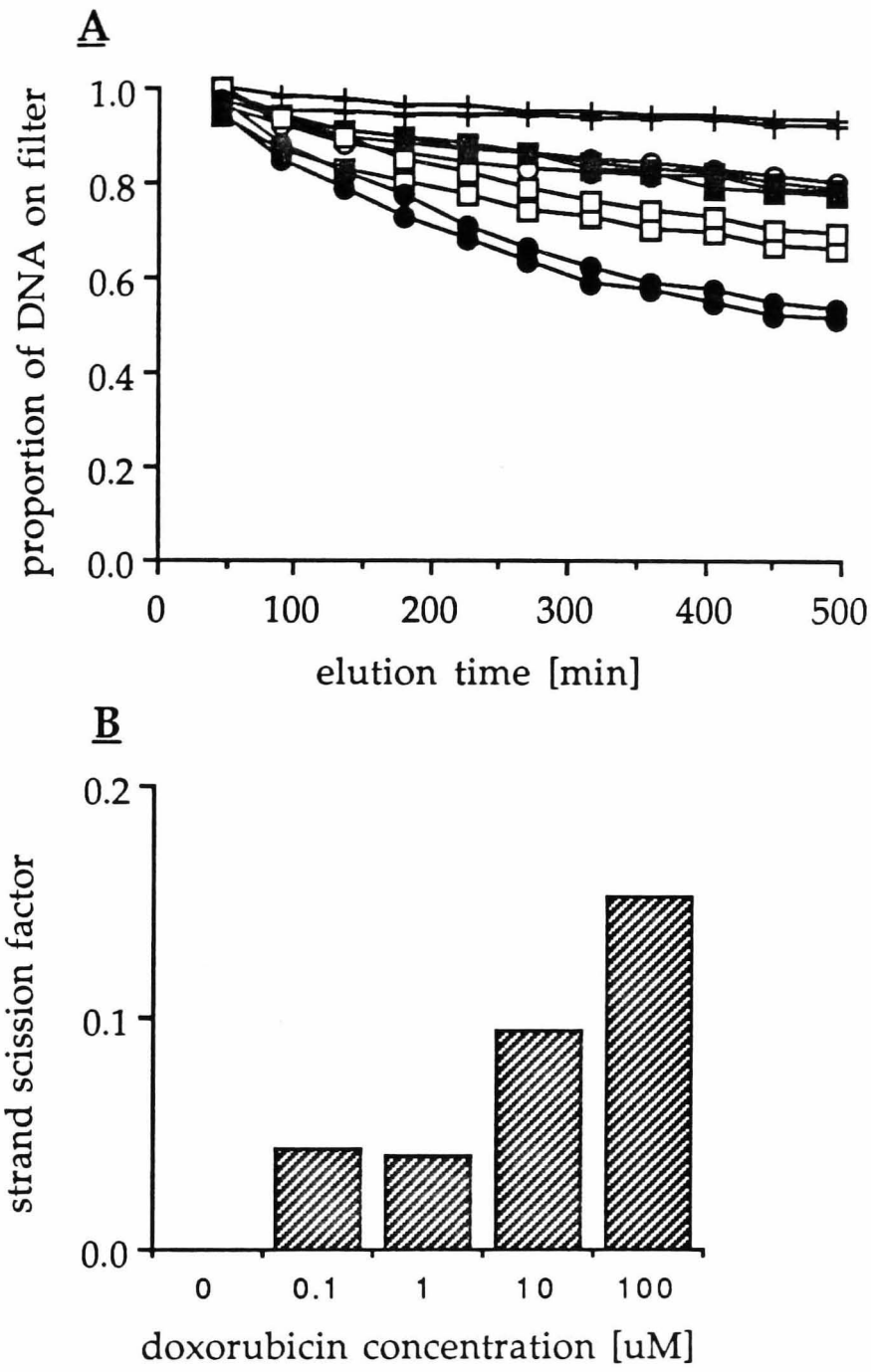


Figure 3.12 A: *Elution rate of DNA isolated from MCF-7 cells treated with doxorubicin.*

MCF-7 cells [$5x10^5 ml^{-1}$] were untreated [+] or treated with doxorubicin 0.1uM [○], 1.0uM [■], 10uM [□] and 100uM [●] for one hour at 37C.

B: *Strand scission factors for doxorubicin derived from A:*

SSF = - log (F_x/F_c) where F_x= proportion of DNA remaining on filter after midpoint elution volume (8.5ml) for treated cells and F_c= as F_x for untreated cells.

cells treated with ED50 concentrations of mitozantrone [$5x10^{-9}M$] or CI941 [$2x10^{-10}M$] for one hour. Mitozantrone produced a more rapid elution rate than CI941 indicative of more strand breakage by mitozantrone than CI941 ar equitoxic concentrations. Figure 3.14 summarises the strand scission factors for mitozantrone and CI941 derived from figures 3.13A and 3.13B. There was no significant difference between the amount of strand scission caused by 100uM and $5x10^{-9}M$ mitozantrone [a 20,000 fold difference in concentration]. In contrast, 60% less strand scission was observed after treatment with $2x10^{-10}M$ compared to 100uM CI941.

3.3.4.3 *Effect of alkylaminoanthraquinones on cellular DNA*

The elution profile of DNA isolated from MCF-7 cells treated with alkylaminoanthraquinones [100uM] for one hour is shown in figure 3.15A. The 1AQ, 1,5AQ and 1,8AQ produced a rapid elution of DNA within the first 100 minutes, representing up to 80% of the total cellular DNA. This was indicative of frank DNA strand breakage such that the DNA fragments produced passed rapidly through the filter on denaturation with alkali. After 100 minutes, only a small amount of the DNA remaining on the filter was further eluted indicating that this DNA was undamaged. In contrast, the elution profile for 1,4AQ was not as rapid but occurred steadily over the elution period. After 100 minutes the amount of DNA eluted from cells treated with 1,4AQ was not significantly different to the amount of DNA eluted from untreated cells.

Strand breakage by the AQ's in MCF-7 cells was further investigated at equitoxic concentrations [ED50 concentrations]. Figure 3.16 shows the elution profile of DNA isolated from MCF-7 cells treated with 1AQ [4uM], 1,4AQ [1.2uM], 1,5AQ [12.3uM] and 1,8AQ [0.5uM] (ED50 concentrations determined in section 4.3.1.4). A similar elution rate was observed for all four AQ's at these concentrations. The strand scission factors for the AQ's, derived from figures 3.15 and 3.16 are shown in figure 3.17. The 1AQ, 1,5AQ and 1,8AQ [100uM] produced a similar amount of strand scission. The amount of strand scission caused by 1,4AQ was

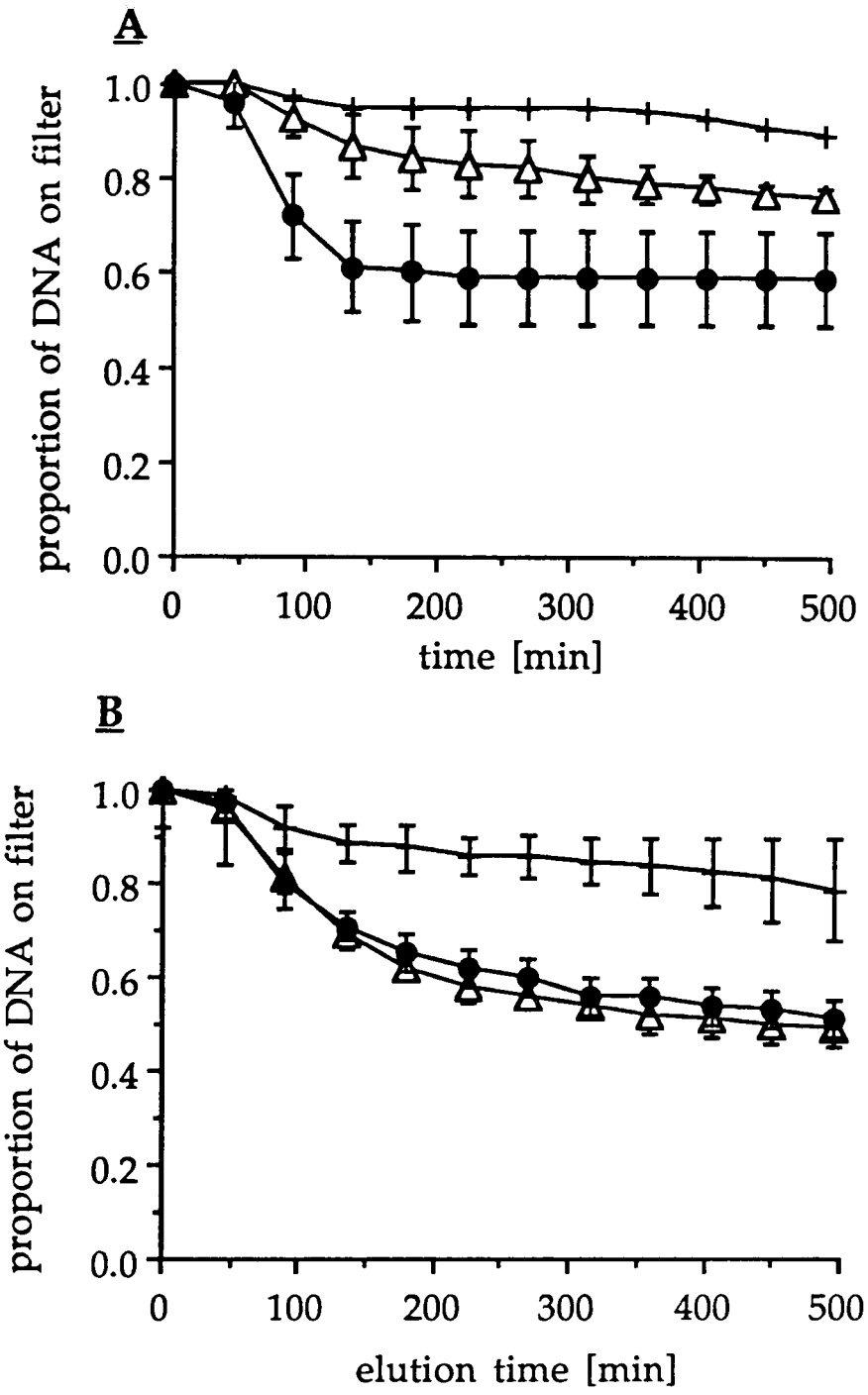


Figure 3.13 *Elution profile of DNA isolated from MCF-7 cells treated with mitozantrone or CI941.*

A: MCF-7 cells [$5x10^5ml^{-1}$] were untreated [+] or treated with mitozantrone ($5x10^{-9}M$) [●] or CI941 ($2x10^{-10}M$) [△] for one hour at 37C.

B: MCF-7 cells [$5x10^5ml^{-1}$] were untreated [+] or treated with mitozantrone (100uM) [●] or CI941 (100uM) [△] for one hour at 37C. Data points are the mean + sd of three determinations.

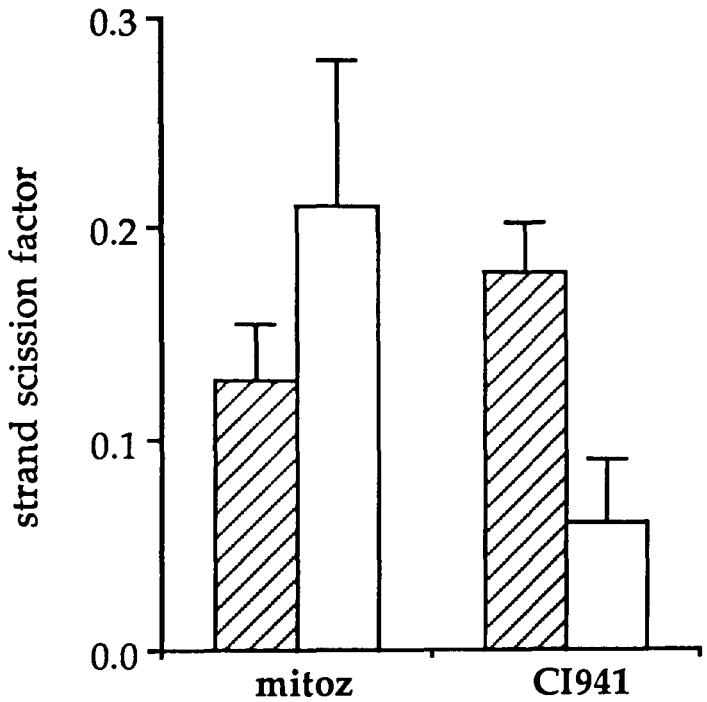


Figure 3.14 *Effect of mitozantrone and CI941 on strand scission of MCF-7 cell DNA as determined by alkaline elution.*

MCF-7 cells were treated (1hr, 37C) with either 100uM [▨] or ED50 [□] concentration of drug. (ED50 mitozantrone = 5nM, ED50 CI941 = 0.2nM). Values are the mean + sd of three determinations. SSF's calculated from figure 3.13A and 3.13B.

significantly less than the other AQ's at this concentration. 1AQ, 1,5AQ and 1,8AQ produced significantly less strand scission at ED50 concentrations compared to 100uM. In contrast, 1,4AQ produced a similar level of strand scission at both concentrations. The amount of strand scission by all four AQ's was not significantly different after treatment with ED50 concentrations, indicating that at equitoxic concentrations the AQ's produce a similar amount of strand breakage.

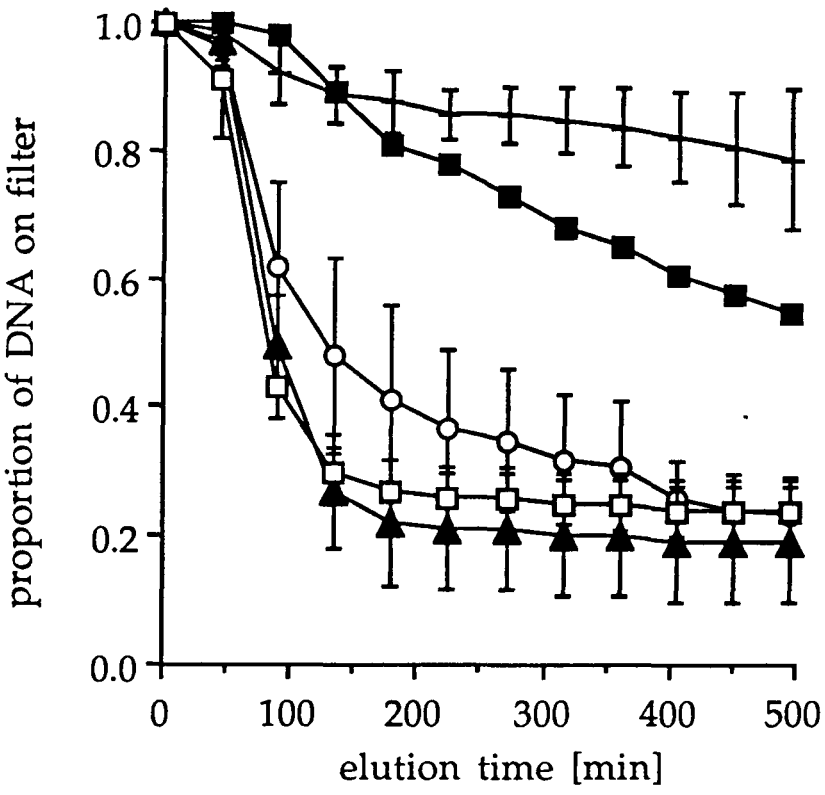


Figure 3.15 *Elution profile of DNA isolated from MCF-7 cells treated with alkylaminoanthraquinones.*

MCF-7 cells [$5x10^5ml^{-1}$] were treated (1hr, 37C) with 100uM of 1AQ [○]. 1,4 AQ [■], 1,5 AQ [▲] or 1,8 AQ [□]. Data points are the mean + sd of four determinations from two experiments.

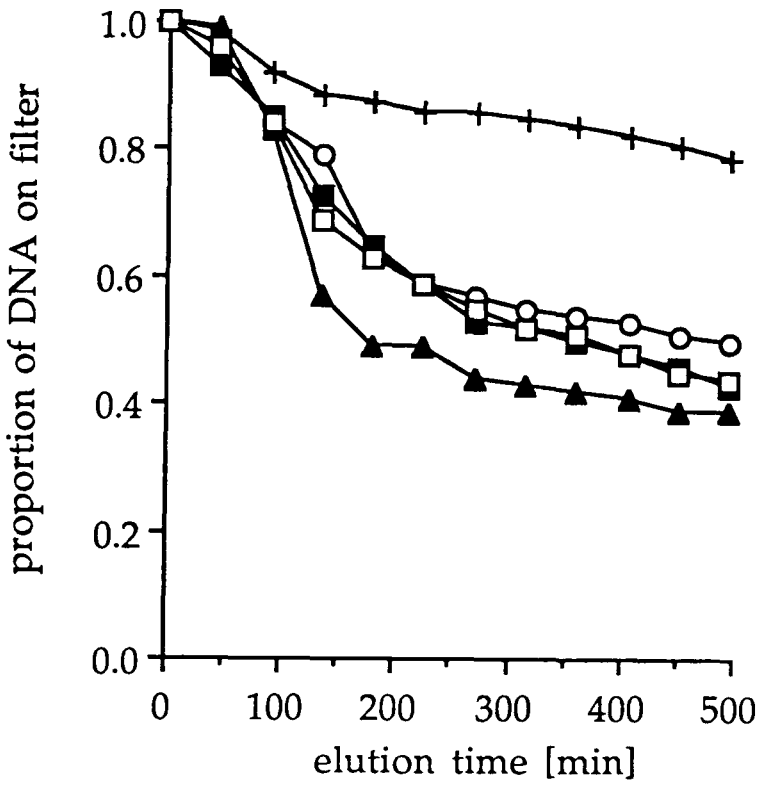


Figure 3.16 *Elution profile of DNA isolated from MCF-7 cells treated with ED50 concentrations of alkylaminoanthraquinones.*

MCF-7 cells [$5x10^5 ml^{-1}$] were treated (1hr, 37C) with [○] 1AQ ($4.0x10^{-6}$ M), [■] 1,4 AQ ($1.2x10^{-6}$M),[▲] 1,5 AQ ($12.3x10^{-6}$M) or [□] 1,8 AQ ($0.5x10^{-6}$M). Data points are the mean + sd of four determinations form two experiments.

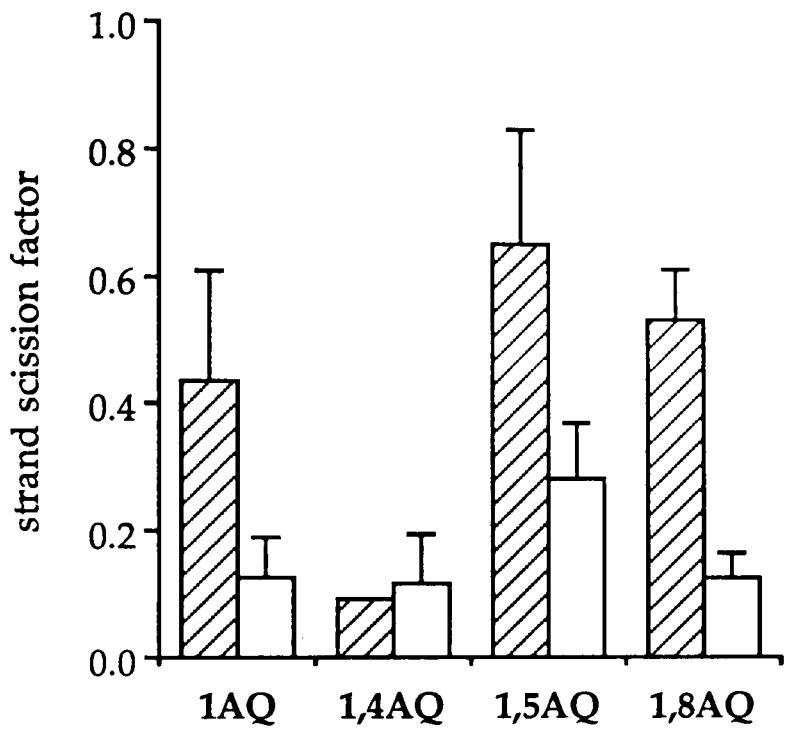


Figure 3.17 *Effect of alkylaminoanthraquinones on strand scission of MCF-7 cell DNA as determined by alkaline elution.*

MCF-7 cells [$5x10^5 ml^{-1}$] were treated (1hr, 37C) with either 100uM [□] or ED50 concentration [□] of drug. Data points are mean + sd of four determinations from two experiments. Strand scission factors calculated from figures 3.15 and 3.16.

3.4 Discussion

3.4.1 *Effect of quinone antitumour agents on strand breakage of plasmid DNA in the presence of purified reductase enzymes.*

The previous chapter has shown that the antitumour agents in this study have different potentials to undergo redox cycling in MCF-7 S9 cell fraction. In this chapter, two purified reductase enzyme systems, namely NADH xanthine oxidase and NADPH cytochrome P450 reductase, were investigated to see if they could reductively activate these agents. Furthermore, the potential of this activation process to instigate DNA strand breakage was studied. The pBR322 plasmid strand breakage assay [described in section 1.12.3] offers a unique opportunity to study both enzymic activation of quinone antitumour agents and whether this activation can mediate DNA strand breakage.

3.4.1.1 *Effect of doxorubicin on strand breakage of plasmid DNA in the presence of purified reductase enzymes*

Doxorubicin was used to characterise the plasmid strand breakage assay with respect to drug, enzyme and DNA concentration. Doxorubicin was found to cause a concentration dependent single and double strand breakage of plasmid DNA in the presence of NADH xanthine oxidase and NADPH cytochrome P450 reductase [see figure 3.18]. This effect was only produced in the presence of the complete incubation system. This indicates that reduction of doxorubicin by the enzyme is a prerequisite for DNA strand scission. The mechanism of strand breakage is likely to proceed via one electron reduction of doxorubicin to a semiquinone free radical species:

$$Q \longrightarrow Q^{\cdot}$$

This reacts in a diffusion controlled manner with molecular oxygen to form the superoxide anion and regenerate the parent drug:

$$Q^{\cdot} + O_2 \rightleftharpoons Q + O_2^-$$

The parent drug becomes available once again for reductive activation. As described previously [section 1.8.2] this process is known as redox cycling. The formation of superoxide anions can lead to the formation of hydroxyl radicals by the following series of reactions:-
Firstly the spontaneous dismutation of superoxide anions:-

$$O_2^- + O_2^- + 2H^+ \longrightarrow 2H_2O_2$$

Followed by the iron driven Haber Weiss reaction:

$$Fe^{3+} + O_2^- \rightleftharpoons Fe^{2+} + O_2$$

$$Fe^{2+} + H_2O_2 \longrightarrow OH^{\cdot} + OH^-$$

$$\text{net } O_2^- + H_2O_2 \longrightarrow O_2 + OH^- + OH^{\cdot}$$

Iron is likely to be present due to it being a ubiquitous contaminant of laboratory agents. The hydroxyl radical, a highly reactive species has been shown to cause DNA strand scission and damage to lipids and proteins [see section 1.9]. The increase in double strand breaks at higher doxorubicin concentrations is supportive of a random hit mechanism in which the more hydroxyl radicals formed the higher the probability of two single strand breaks occurring on directly opposite DNA strands to produce a double strand break. Iron bound to the DNA or the drug (see section 1.9.2.1) may lead to 'site specific' DNA strand scission [reviewed by Ward, 1987]:

$$DNA\text{-}Fe^{2+} + H_2O_2 \longrightarrow DNA\text{-}Fe^{3+} + OH^{\cdot} + OH^-$$

$$OH^{\cdot} + DNA \longrightarrow DNA\text{-SSB (single strand break)}$$

$$DNA\text{-}Fe^{3+} + O_2^- \rightleftharpoons DNA\text{-}Fe^{2+} + O_2$$

$$DNA\text{-}Fe^{2+} + H_2O_2 \longrightarrow DNA\text{-}Fe^{3+} + OH^{\cdot} + OH^-$$

$$DNA\text{-SSB} + OH^{\cdot} \longrightarrow DNA\text{-DSB (double strand break)}$$

DNA strand breakage could also occur by covalent binding of the doxorubicin free radical to the DNA [Sinha and Chignell, 1979]. Hydroxyl radical attack on DNA has been proposed to be due to hydrogen abstraction from the 2', 3' or 4' carbon atoms of deoxyribose [see figures 3.19 and 3.20]. This results in formation of a deoxyribose radical which may be attacked by molecular oxygen to form a peroxy radical. This can abstract a hydrogen atom from a neighbouring DNA sugar or base to give a hydroperoxide. Therefore, the radical species may be propagated along the DNA molecule. The hydroperoxide can decompose in the presence of transition metals to give a hydroperoxide free radical, alternatively the hydroperoxide can eliminate water to produce breakage of the sugar ring followed by hydrolysis of this modified sugar to generate a strand break (Figure 3.20) [reviewed by Tanaka, 1984]. Alternatively the carbon-carbon double bond of the DNA bases are a target for hydroxyl radical attack resulting in a strand break or base modification and base loss (figures 3.21 and 3.22) [see section 1.9.2.2] (reviewed by Von Sonntag, 1988). Oxygen free radical attack on pBR322 DNA has been shown to result in the formation of endonuclease sensitive sites due to the production of thymine glycol residues (see figure 3.22) [Denq and Fridovich, 1989]. The findings of this present study are supported by the work of other investigators. Bates and Winterbourn (1982) also showed that activation of doxorubicin by xanthine oxidase could mediate strand breakage of DNA, this being inhibited by superoxide dismutase and catalase. DNA strand breakage produced in a system of doxorubicin and NADPH cytochrome P450 reductase has been shown to be dependent on the presence of molecular oxygen (Berlin and Haseltine, 1981). Furthermore, this DNA strand breakage was partially inhibited by superoxide dismutase and catalase (Lown *et al.*, 1977, Someya and Tanaka, 1979). Furthermore, in the presence of cytochrome P450 reductase doxorubicin has been shown to stimulate NADPH oxidation and form an esr detectable free radical species (Bachur *et al.*, 1979; Berlin and Haseltine, 1981).

The results obtained in the present study provide further evidence for doxorubicin undergoing reductive metabolic activation, correlating with the redox cycling activity of this agent in MCF-7 human breast cancer cells (section 2.3.1). Xanthine oxidase (a cytosolic enzyme) and

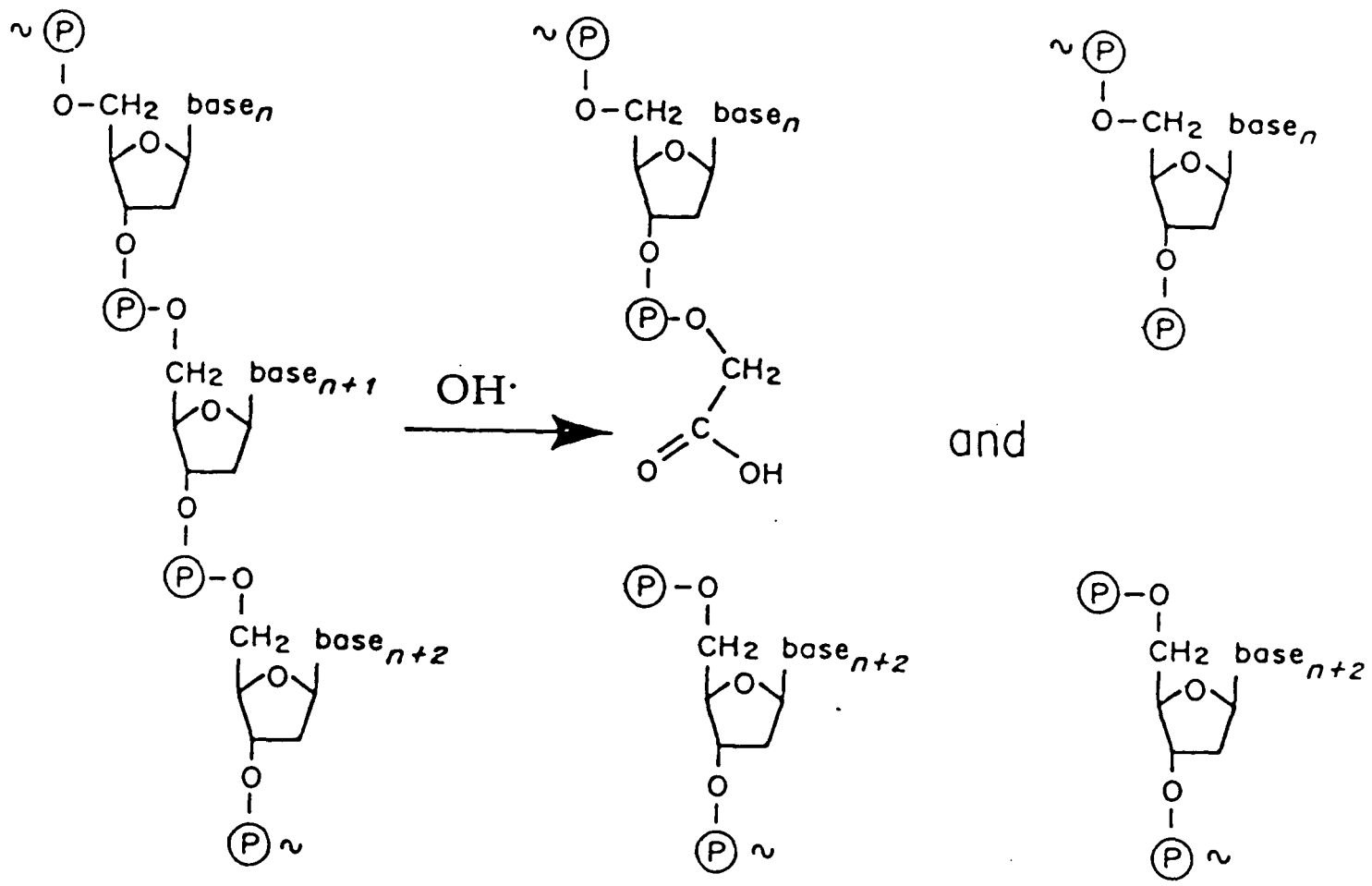


Figure 3.19 *Structure of repairable free radical mediated single strand breaks in DNA*

Products are the 3′-phosphoglycolate residue or total destruction of nucleotide n+1 (Teebor *et al.*, 1988)

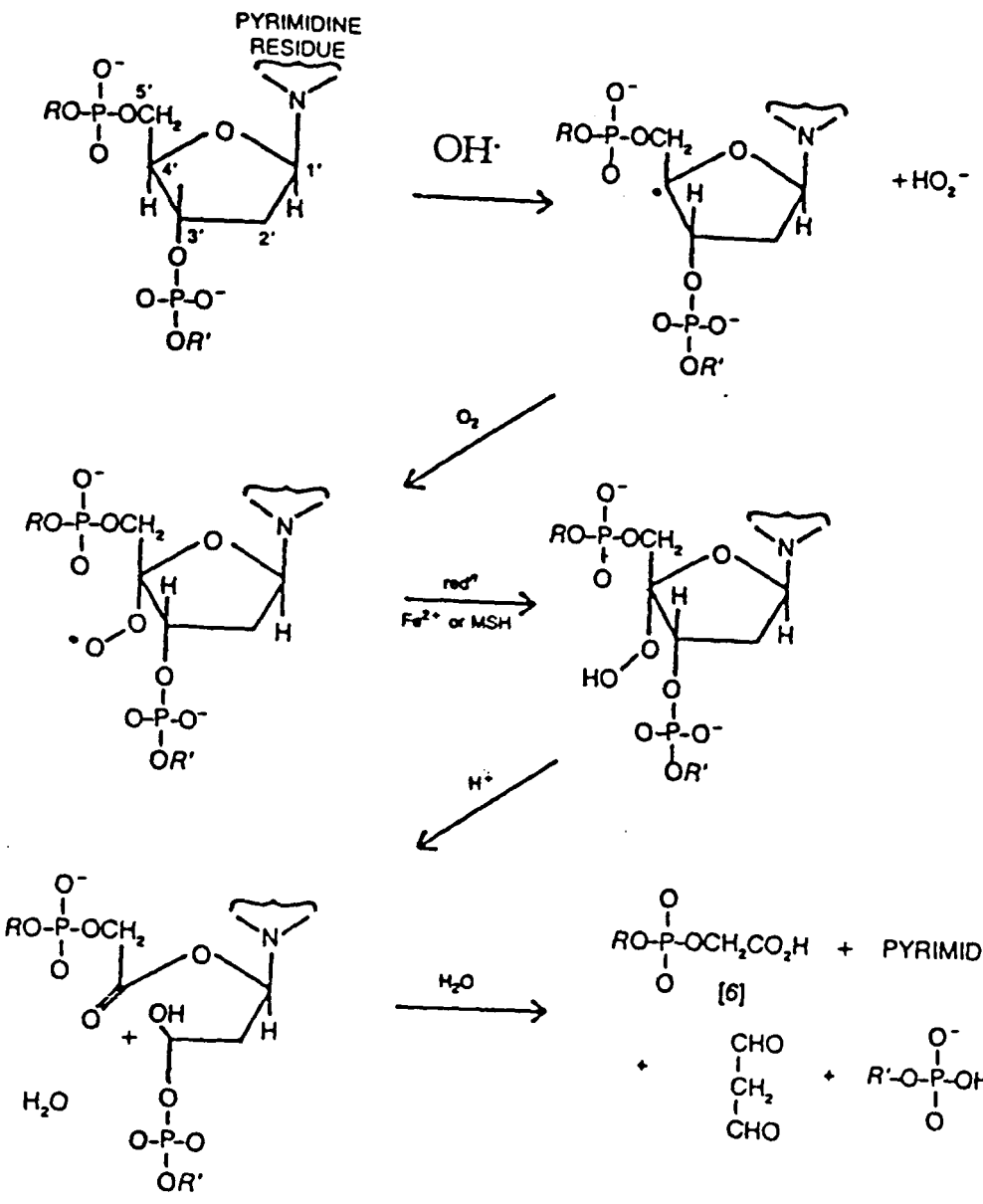


Figure 3.20 *Postulated mechanism for hydroxyl radical mediated cleavage of DNA*

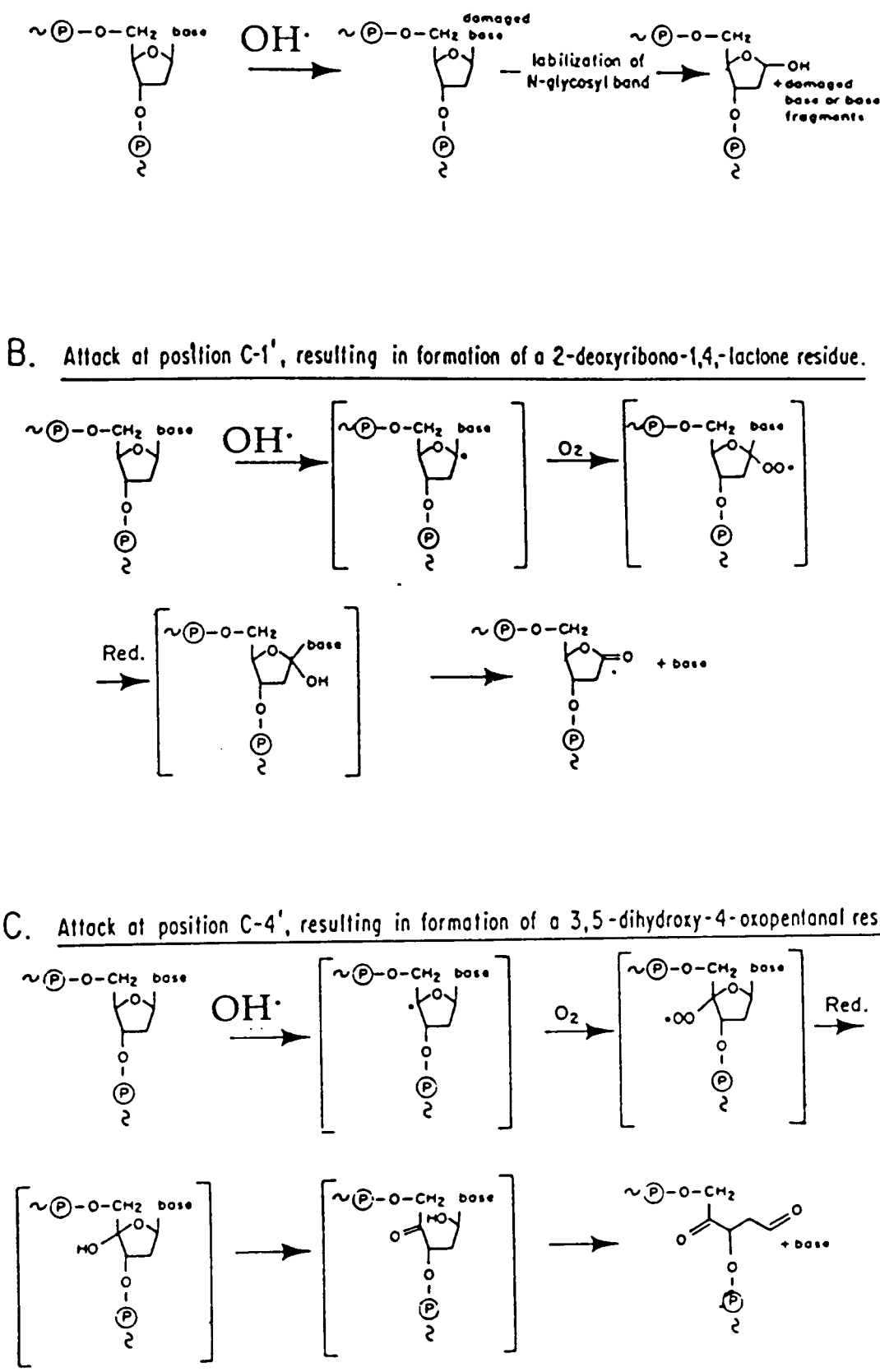


Figure 3.21 *Mechanism of hydroxyl radical mediated formation of an apurinic/apyrimidinic site on DNA*

A. Attack on the base results in labilisation of the bond and release of the damaged base, leaving a deoxyribose residue in the DNA backbone.
B, C. These are the best characterised residues resulting from free radical attack on the sugar moiety. The structures within brackets are intermediates thought to lead to the final product.

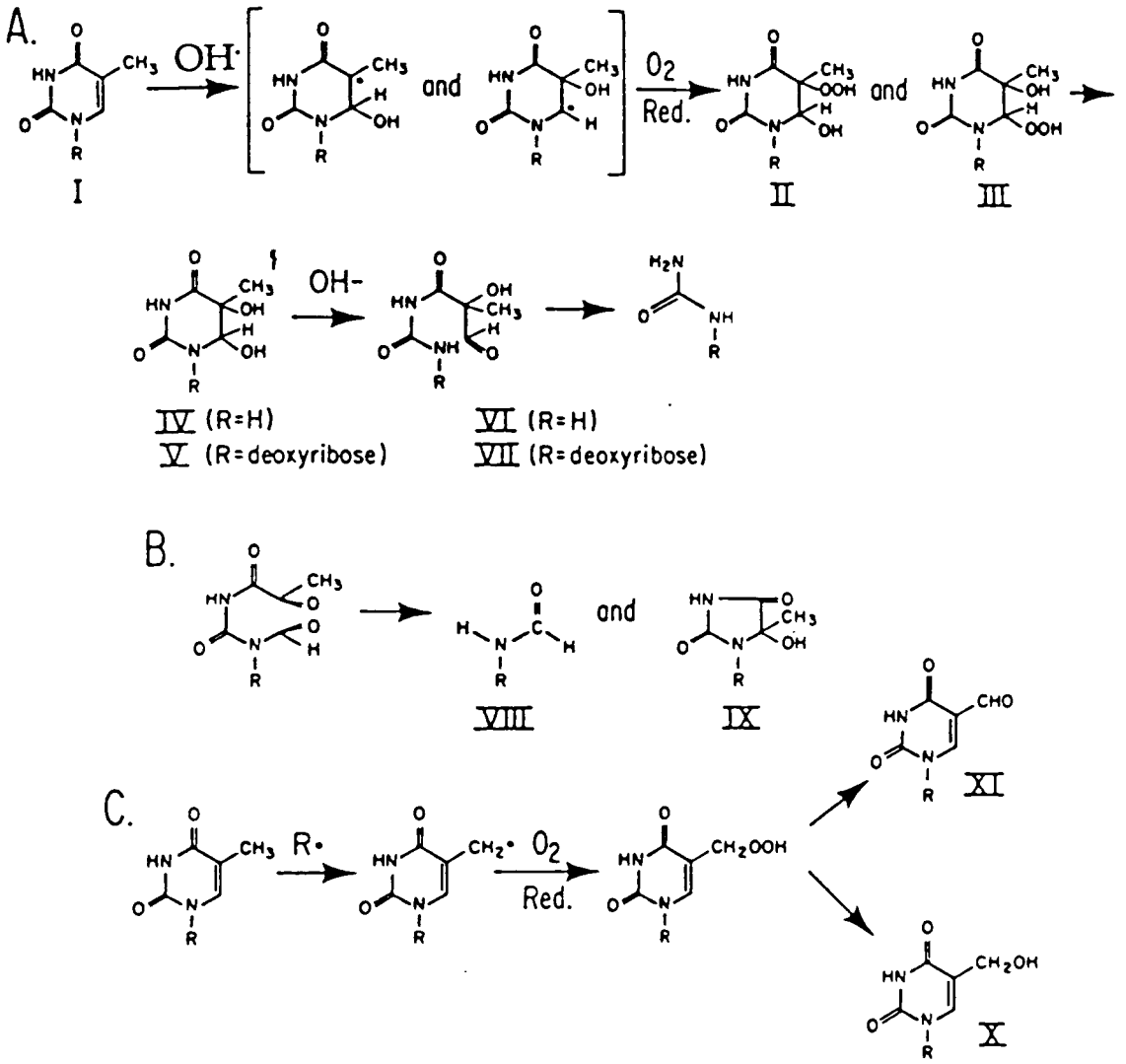


Figure 3.22 *Hydroxyl radical mediated degradation of thymidine.*

A. This reaction sequence results from attack on the 5,6 ethylenic bond, which in the presence of oxygen, leads to formation of hydroperoxides which may be reduced to the repairable thymine glycol moiety.
B. Oxidation and fragmentation of the 5(6)-hydroxy-(6)5-peroxides result in formation of formamide and 5-hydroxy-5-methyl hydantoin residues both of which are repairable.
C. This reaction sequence illustrates the products derived from attack on the exocyclic methyl group leading to formation of the repairable 5-hydroxymethyluracil moiety and the 5-formyluracil moiety whose repairability is uncertain.

cytochrome P450 reductase (a microsomal enzyme) are therefore possibly involved in the reductive activation of doxorubicin in this cellular system. These results also indicate that activation of doxorubicin in this manner can mediate DNA strand breakage.

3.4.1.2 *Effect of mitozantrone and CI941 on strand breakage of plasmid DNA in the presence of purified reductase enzymes*

In contrast to doxorubicin, mitozantrone produced only a small amount of DNA strand breakage in a system containing NADH xanthine oxidase [figure 3.3] and CI941 produced no significant effect [figure 3.3]. This result indicates that these compounds are poor substrates for reductive metabolic activation by this enzyme. In a system of NADPH cytochrome P450 reductase, mitozantrone produced a dose dependent single and double strand breakage of plasmid DNA whereas CI941 showed no significant effect [see figure 3.4]. The inability of CI941 to produce strand breakage in the presence of cytochrome P450 reductase is consistant with CI941 not being reductively activated by this enzyme as shown by Graham *et al.* (1987). As previously described for doxorubicin [section 3.4.1.1] the mechanism of mitozantrone mediated strand breakage in this system is likely to involve reactive oxygen species. Oldcorne *et al.* (1985) also showed that mitozantrone produced DNA strand breakage in the presence of cytochrome P450 reductase. In addition Kharasch and Novak (1983a) showed that mitozantrone formed superoxide anions in the presence of this enzyme.

3.4.1.3 *Effect of alkylaminoanthraquinones on strand breakage of plasmid DNA in the presence of purified reductase enzymes*

A series of mitozantrone related alkylaminoanthraquinones [figure 1.35] were also investigated with respect to their propensity to mediate plasmid DNA strand breakage in the presence of purified xanthine oxidase and cytochrome P450 reductase. This was in order to assess the effect of the side chain substitution pattern on their relative propensity for

reductive activation. None of the AQ's produced significant strand breakage in the presence of NADH xanthine oxidase indicating that these compounds are not substrates for reductive activation by this enzyme (figure 3.5A). However, the AQ's produced different amounts of DNA single strand breaks in the presence of NADPH cytochrome P450 reductase (figure 3.5B). 1AQ, 1,5AQ and 1,8AQ all produced single strand breakage of plasmid DNA though to a lesser extent than doxorubicin or mitozantrone. This indicates that these compounds are substrates for reductive activation by this enzyme. DNA strand breakage is likely to be mediated via production of reactive oxygen species in a redox cycling process as described for doxorubicin [section 3.4.1.1]. In contrast, the 1,4AQ produced negligible strand breakage indicating that this compound is a poor substrate for reductive activation by cytochrome P450 reductase. From these results the AQ's can be ranked in order of propensity for activation by NADPH cytochrome P450 reductase:- 1AQ = 1,5AQ = 1,8AQ > 1,4AQ.

To summarise, the results presented in this section show that the quinone antitumour agents in this study have markedly different propensities to undergo reductive activation by purified flavin reductase enzymes. This activation results in the formation of reactive oxygen species by the process of redox cycling which mediate DNA strand breakage. A role for reactive oxygen in strand breakage is supported by the similar profile of plasmid DNA strand scission produced by gamma irradiation (appendix figure A4) and hydrogen peroxide (appendix figure A5) which are proposed to mediate DNA strand scission via hydroxyl radical formation (reviewed by Ward, 1987; Floyd and Lewis, 1983). In contrast CI941, was not a substrate for reductive activation by these enzymes. Mitozantrone was not activated by xanthine oxidase but was activated by cytochrome P450 reductase. Similarly, the AQ's (except 1,4AQ) were activated by cytochrome P450 reductase. The relative propensities of mitozantrone, CI941 and AQ's to undergo reductive activation correlates well with their one electron reduction potentials [table 2.5] but is also likely to be influenced by their structural differences, including the positions of the alkylamino side chain on the anthraquinone chromophore. As discussed in section 2.4.2 this is possibly due to steric hindarance of the interaction between the

anthraquinone chromophore and the flavin component of the reductase.

3.4.2 *The effect of antitumour agent-iron complexes on plasmid DNA.*

Doxorubicin has been shown to bind iron with a high degree of affinity, a phenomenon that has only recently gained interest (reviewed by Garnier-Suillerot, 1988). As described in section 1.8.3 iron is proposed to play an intimate role in the mediation of cell damage by drugs that redox cycle, resulting in superoxide anion formation. This is due to the involvement of iron in the iron catalysed Haber-Weiss reaction (see section 1.8.3) which results in the production of hydroxyl radicals from superoxide anions and H_2O_2. Drug bound iron has been found to undergo a non-enzymic redox cycle mechanism in the presence of cellular thiols [eg reduced glutathione (GSH)] by the following series of reactions (reviewed by Myers *et al.*, 1986):-

$$2\text{Drug-Fe}^{3+} + 2\text{GSH} \longrightarrow 2\text{Drug-Fe}^{2+} + \text{GSSG (oxidised GSH)} \quad (1)$$

$$\text{Drug-Fe}^{2+} + O_2 \rightleftharpoons \text{Drug-Fe}^{3+} + O_2^{-} \quad (2)$$

$$O_2^{-} + O_2^{-} + 2H^{+} \longrightarrow H_2O_2 \quad (3)$$

$$O_2^{-} + \text{Drug-Fe}^{3+} \rightleftharpoons \text{Drug-Fe}^{2+} + O_2 \quad (4)$$

$$\text{Drug-Fe}^{2+} + H_2O_2 \longrightarrow \text{Drug-Fe}^{3+} + OH^{-} + OH\cdot \quad (5)$$

These reactions, analagous to the those that produce hydroxyl radicals during reductase mediated quinone redox cycling [section 1.8.3] are independent of the potential of the quinone to undergo enzyme mediated redox cycling. In this study the ability of a doxorubicin-iron complex to cause DNA strand breakage in the presence of reduced glutathione was investigated using the plasmid DNA strand breakage assay.

A doxorubicin-iron complex formed from mixing doxorubicin and ferrous iron (1:2), in the presence of reduced glutathione, produced a

concentration dependent decrease in DNA recoverable from the incubation, as well as some double strand breaks [see figure 3.6]. The absence of strand breakage in the presence of ferrous iron only [figure 3.6] would suggest that all this iron has been rapidly oxidised to ferric before the start of the incubation (Myers *et al.*, 1982). The loss of DNA observed was probably due to frank strand breakage resulting in the formation of low molecular weight DNA fragments. These are either not recovered by the DNA precipitation and centrifugation step during the assay procedure or are not resolved by gel electrophoresis. The DNA strand breakage observed is likely to take place via a mechanism involving the production of reactive oxygen species as described above (reactions 1-5). Doxorubicin has been shown to form a 3:1 complex with Fe^{3+} (Beraldo *et al.*, 1985) with stepwise association constants of 10^{18}, 10^{11} and $10^{4.4}$ (May *et al.*, 1980), which can intercalate into DNA releasing the iron or produce a ternary drug-Fe^{3+}-DNA complex due to the high affinity of Fe^{3+} for DNA phosphate groups (reviewed by Garnier-Suillerot 1988). The doxorubicin-iron complex involves the coordination of one phenolate C_{11} oxygen and one C_{12} carbonyl from each drug molecule to Fe^{3+} to give a six membered chelate [see figure 3.23]. In agreement with the results of this study, a doxorubicin-Fe^{3+} complex has been shown to cause destruction of erythrocyte ghost cell membranes with concomitant consumption of oxygen in the presence of glutathione. This was process totally inhibited by a combination of SOD and partially blocked by the hydroxyl scavenger mannitol or the iron chelator EDTA (Myers *et al.*, 1982). This effect was greater when the drug-iron-DNA complex was used (Myers *et al.*, 1982). This is presumably due to the stabilisation of the iron by this ternary complex. Also, Eliot *et al.* (1984) showed that a doxorubicin-Fe^{3+} complex caused strand breakage of DNA in the presence of glutathione. A role for hydroxyl radical involvement in DNA-bound doxorubicin-iron complex mediated DNA damage is further supported by the work of Muindi *et al.* (1984) who found that a DNA-Fe^{3+}-doxorubicin ternary complex was more effective than complex alone at producing hydroxyl radicals in the presence of glutathione. The system used in the present study initially involves formation of a doxorubicin-Fe^{2+} complex. The Fe^{2+} is likely to be rapidly oxidised to Fe^{3+} by molecular oxygen with reduction of the oxygen to superoxide anions (equation 2). This part of the reaction is glutathione independent. However, the doxorubicin-Fe^{3+} formed becomes available for

reduction by glutathione (equation 1). Doxorubicin-Fe^{2+} can either react with molecular oxygen to form superoxide anions or take part in the iron-catalysed Haber-Weiss reaction with resultant production of hydroxyl radicals from superoxide anions and hydrogen peroxide [reactions 2, 4 and 5]. It is likely that DNA strand breakage is produced by a combination of these processes, the reactive species involved being hydroxyl radicals. The reactions shown above [equations 1-5] are likely to be an over simplification of the mechanisms involved, as participation of a perferryl radical species (Fe^{3}-O_2^{-}) cannot be discounted (reviewed by Halliwell and Gutteridge, 1985). Mechanisms of reactive oxygen attack on DNA are described in section 1.9.2 and 3.4.1.1 (see figures 3.19-3.21).

By analogy with the structural similarity to doxorubicin mitozantrone would appear to have structural requirements necessary for iron binding. Indeed mitozantrone has been shown to form complexes with copper (II) and platinum (II) ions (reviewed by Garnier-Suillerot, 1988). However, no studies have been carried out on the interaction of iron with mitozantrone, the alkylaminoanthraquinones or the anthrapyrazoles. This study has investigated the ability of these agents in combination with iron to generate plasmid DNA strand breakage.

A complex generated from a 1:2 mixture of mitozantrone and ferrous iron, in the presence of glutathione, was found to produce double strand breakage of plasmid DNA, whilst drug or iron alone produced no effect [see figure 3.7]. A 1:2 drug:iron ratio was used as mitozantrone has two possible iron binding sites between the aromatic hydroxyl groups and the carbonyl groups. The mitozantrone-iron complex proved difficult to extract from the DNA incubate despite the presence of desferrioxamine, a strong iron chelator. This indicates that the complex is tightly bound to DNA, possibly in the form of a ternary complex involving mitozantrone-Fe^{3+}-DNA. The absence of DNA strand breakage in the presence of Fe^{2+} alone shows that the presence of a mitozantrone-iron complex is a prerequisite. This is probably due to the strong affinity of mitozantrone for DNA (see section 1.17) which targets the iron to a site closely adjacent to the DNA strands. In a similar manner to doxorubicin, the mitozantrone-Fe^{2+} complex initially produced is likely

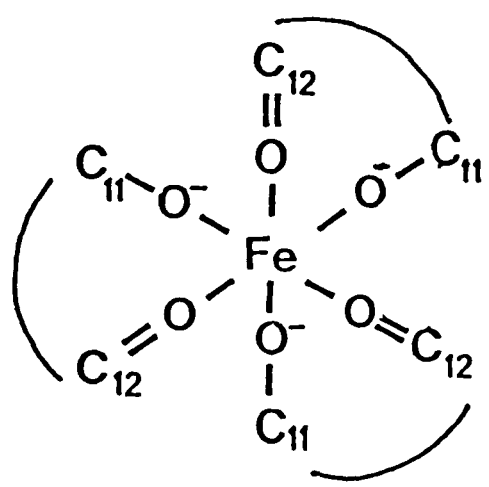


Figure 3.23 *Schematic diagram of the Fe(doxorubicin)$_3$ complex.*

to have rapidly oxidised to mitozantrone-Fe^{3+} with concomitant reduction of molecular oxygen (equation 2). The superoxide anion formed in this manner could participate in DNA strand breakage via hydroxyl radical formation (reactions 3-5). The results of this study indicate that mitozantrone forms an iron complex which can mediate DNA damage in the presence of reduced glutathione. The likely iron binding sites on mitozantrone are between the aromatic hydroxyl groups [C_5 and C_8] and their adjacent carbonyl groups. The role of these hydroxyl groups in iron binding is supported by the lack of DNA strand breakage produced by 1:2 mixtures of alkylaminoanthraquinones and ferrous iron [see figure 3.10]. This suggested an absence of iron binding by these agents which do not possess C_5 and C_8 aromatic hydroxyl groups.

The anthrapyrazoles [APZ] are antitumour agents which have recently been developed [see section 1.15]. Structurally they are similar to mitozantrone, however, the quinone moiety is replaced by a quasi-iminoquinone. This modification was carried out to reduce their interaction with reductase enzymes, hence diminish their ability to redox cycle. Despite this modification it is likely that iron binding can occur between the C_7 and C_{10} hydroxy groups and the adjacent imino or carbonyl groups. A series of APZ's [figure 1.36] including CI941 (7-OH APZ) with modified chromophores at the C_7 and/or C_{10} hydroxyl groups were investigated with respect to produce DNA strand breakage in combination with ferric iron in the presence of glutathione. In these studies ferric iron was used to produce APZ-iron complexes in order to circumvent direct involvement of Fe^{2+} oxidation in reactive oxygen formation and strand breakage. Therefore, formation of APZ-Fe^{2+} should be anticipated to be primarily as a result of glutathione reduction of APZ-Fe^{3+} in this system. In the presence of glutathione and a combination of APZ's and ferric iron, only the C_{10} hydroxyl group substituted 7,10-OH-APZ and 10-OH-APZ produced single and double strand breaks in plasmid DNA [see figure 3.9 and photograph 3.3]. This strand breakage is likely to be mediated by the series of reactions [1-5] described above with resultant hydroxyl radical formation. No strand breakage was observed for these compounds in the absence of glutathione or in the presence of iron or drug alone. This indicates that it is the ferric iron coordinated at the C_{10} hydroxyl group and the imino group which is participating in glutathione mediated redox cycling of iron.

The absence of DNA strand breakage by the 7,10-H APZ correlates with the requirement of the aromatic hydroxyl groups for iron binding. The APZ's investigated in the present study have previously been shown to bind ferric iron (Graham *et al.*, 1989). However, 7-OH APZ (CI941) only weakly complexed ferric iron and 7,10-H APZ did not bind ferric iron. This would indicate that absence of DNA strand breakage by 7-OH APZ (CI941) in combination with ferric iron is due to it being only a weak binder of this iron species. In nitrogen purged incubates the 7,10-OH APZ and 10-OH APZ produced an equivalent amount of DNA strand breakage as in the presence of oxygen. Despite making all efforts to remove oxygen from the system it is likely that sufficient oxygen was present to allow reactive oxygen mediated strand breakage to occur.

This study has shown that quinone antitumour agents with aromatic hydroxyl groups can form iron complexes which produce DNA strand breakage in the presence of glutathione by a mechanism likely to involve formation of reactive oxygen species. As discussed in section 3.4.1, a role for oxygen free radical species in quinone-iron complex mediated plasmid DNA strand breakage is supported by the similar pattern of strand breakage of plasmid DNA produced by gamma radiation (appendix figure A4) and hydrogen peroxide (appendix figure A5). Iron has been implicated in hydrogen peroxide mediated DNA scission (Floyd and Lewis, 1983). Binding to DNA is likely to be an important contributory factor to DNA strand breakage by drug-iron complexes presumably due to the glutathione mediated redox cycling of iron and formation of reactive oxygen species proximal to DNA.

The results of this study could have important implications in the mechanism of action of these agents. Clinical use of such complexes seems unlikely since it has been shown that at physiological pH and at clinical drug concentrations doxorubicin would have very little iron bound even if it is injected as a complex (Gelvan and Samuni, 1988). In neutral aqueous solution the concentration of soluble ferric iron is only 10^{-11}-10^{-18}M (reviewed by Myers *et al.*, 1986). It has been proposed that doxorubicin at clinically relevant concentrations will not bind adventitious iron. Clinical trials with quelamycin a doxorubicin-iron chelate produced conflicting results as to its efficacy and cardiotoxicity [reviewed by Garnier-Suillerot, 1988].

3.4.3 *Effect of quinone antitumour agents on topoisomerase activity in MCF-7 cell nuclear extract*

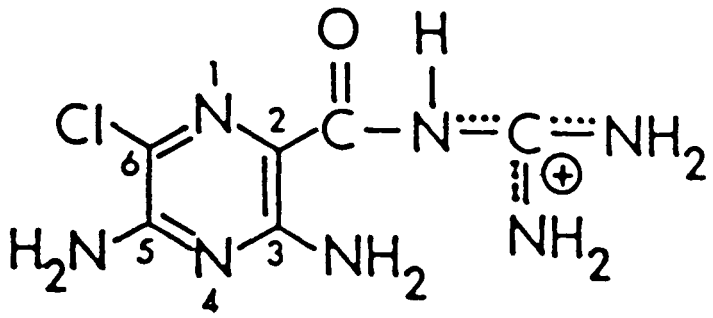
3.4.3.1 *The effect of doxorubicin, topoisomerase II and topoisomerase I inhibitors on topoisomerase activity in MCF-7 cell nuclear extract*

Recently the mechanism of action of some DNA binding agents has been related to their ability to inhibit the nuclear enzymes topoisomerase I and II [reviewed by Ross, 1985 and Lock and Ross, 1987) [see section 1.7.1]. These enzymes are involved in controlling the topological state of DNA. The topoisomerases catalyse knotting/unknotting, relaxation/supercoiling and catenation/decatenation of DNA [see figures 1.13 and 1.14] (reviewed by Maxwell and Gellert, 1986). The difference between the two types of topoisomerase is that topoisomerase I changes the linking number of DNA by one whilst topoisomerase II changes it by two. These changes are brought about by transient strand breaks either single [topoisomerase I] or double [topoisomerase II] which allow passage of either a single DNA strand or a double DNA strand respectively. Mechanistically, the difference between the two enzyme types can be discriminated by the requirement of ATP and magnesium ions for topoisomerase II activity. In the present study the ability of quinone antitumour agents to inhibit topoisomerase activity isolated from MCF-7 human breast cancer cells was investigated. Supercoiled pBR322 plasmid DNA was used as a substrate and the topomers formed resolved by agarose gel electrophoresis. The assay procedure for topoisomerase activity is used to assess two drug induced end points, inhibition of relaxing activity and formation of topoisomerase cleavable complexes. Topoisomerase cleavable complexes [see section 1.7.2 and 1.7.3] are formed due to trapping of the enzyme on the DNA duplex by the presence of drug and are proposed to consist of DNA and enzyme in a conformation which is susceptible to cleavage in the presence of strong protein denaturants such as sodium dodecyl sulphate [SDS] and proteinase K. Denaturation of the cleavable complex reveals either DNA single [topoisomerase I and II] or double [topoisomerase II] strand breaks. In the case of topoisomerase I these breaks are associated with

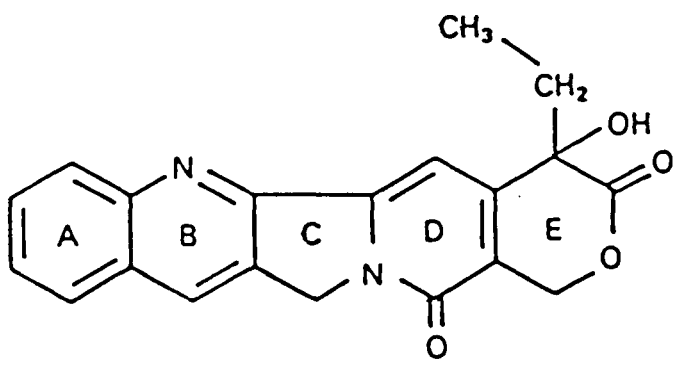
topoisomerase attached to the free 3′ end of the DNA break by a 3′-phosphotyrosine linkage, whereas topoisomerase II enzyme subunits are associated with the 5′ ends of the broken DNA double strands also via tyrosyl linkages [reviewed by Wang, 1985].

Topoisomerase II activity has been shown to be dependent on the proliferative status of the cells with less activity being found in quiescent cells (Sullivan *et al.*, 1987; Markovits *et al.*, 1987; Hsiang *et al.*, 1988). Cellular content and activity of topoisomerase II also varies during the cell cycle (Heck *et al.*, 1988; Hsiang *et al.*, 1988). In contrast, topoisomerase I levels do not vary between various growth states (reviewed by Liu, 1983). A nuclear extract was prepared from MCF-7 cells by the method described by Minford *et al.* (1986). This extract has been reported to contain both topoisomerase I and II activity. The supercoiled DNA relaxing activity of this extract was not dependent on the presence of ATP [photograph 3.4] suggesting that this activity was topoisomerase I. This correlates with the MCF-7 cells used to prepare the nuclear extract being in a non-proliferative (confluent) stage of growth. In order to identify the topoisomerase type present in MCF- 7 nuclear extract, the effect of the topoisomerase II inhibitors mAMSA (Nelson *et al.*, 1984) and amiloride (Besterman *et al.*, 1987) and the topoisomerase I inhibitor camptothecin (Hsiang and Liu, 1988; Hertzberg *et al.*, 1989) on the relaxing activity of MCF-7 nuclear extract was investigated [see figure 3.18 for drug structures].

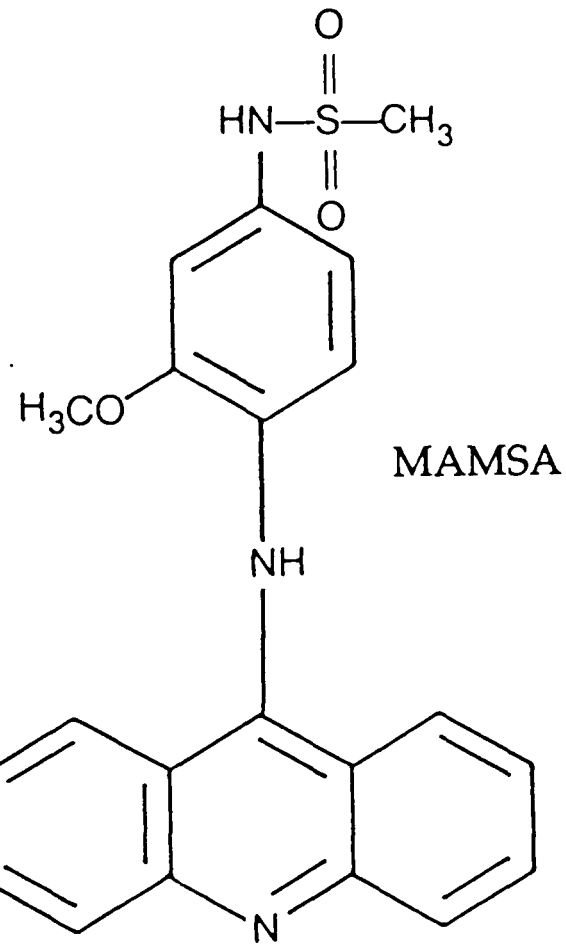
In the presence of topoisomerase I or II the supercoiled plasmid DNA is relaxed to form open circular DNA and intermediate species (topomers) with degrees of unwinding between supercoiled and open circular with differences in linking number of 1 or 2 [see photograph 3.5]. The reaction was terminated with SDS or SDS and proteinase K. In the presence of active topoisomerase I or II inhibitors a reduction in the number of topomers and the amount of open circular DNA should be observed. However, when the reaction was stopped with SDS only mAMSA, amiloride and camptothecin [all 100ng ml^{-1}] had no effect on the above processes [photograph 3.5].



amiloride



camptothecin



MAMSA

Figure 3.18 *The structures of mAMSA, amiloride and camptothecin.*

When the reaction was stopped with SDS and proteinase K mAMSA, amiloride and camptothecin also produced no effect on the DNA relaxing activity of MCF-7 nuclear extract [photograph 3.5]. If these drugs produced topoisomerase-DNA cleavable complexes an increase in linear [topoisomerase II] or open circular DNA [topoisomerase I and II] should be observed under these conditions. These results were inconclusive as to the identity of the type of topoisomerase activity present in MCF-7 nuclear extract. The concentations of these agents required to inhibit topoisomerase activity vary considerably between different laboratories [see table 3.2] therefore the concentrations used in the present study may have been too low. Furthermore, amiloride is reported to be a very weak (optimal concentration 115ugml^{-1}) inhibitor of topoisomerase II and does not form cleavable complexes (Besterman *et al.*, 1987). Therefore, in a further experiment, the effect of doxorubicin [0.1uM] and a higher concentration of mAMSA [10uM, 4.0ugml^{-1}] were investigated on the DNA relaxing activity of the MCF-7 nuclear extract. Doxorubicin has also been reported to be an inhibitor of topoisomerase II activity (Tewey *et al.*, 1984). When the reaction was stopped with SDS and proteinase K, this higher concentration of mAMSA slightly inhibited topomer formation and resulted in the formation of mainly open circular DNA [Photograph 3.6]. This is consistent with topoisomerase I- DNA cleavable complex being formed in the presence of mAMSA, which in the presence of proteinase K was being converted to nicked open circular DNA. Unfortunately this species cannot be discriminated from unnicked open circular in the plasmid assay since both species have identical migration rates through the agarose gel. Previously, mAMSA had been reported to be a specific inhibitor of topoisomerase II activity [Nelson *et al* 1984; Rowe *et al.*, 1986; Minford *et al* 1986; Pommier *et al* 1987] with good correlation between protein associated strand breakage and its cytotoxicity (Bakic *et al.*, 1986; Pommier *et al.*, 1984, 1985, 1986; Per *et al.*, 1987). It has recently been reported that mAMSA is a potent inhibitor of topoisomerase I isolated from L1210 cells (Pommier *et al.*, 1987), although, cleavable complex formation was not observed by these workers. Thus, it is likely that the DNA relaxing activity in MCF-7 nuclear fraction is topoisomerase I due to it being inhibited by mAMSA, producing cleavable complex mediated single strand breakage. If this activity was topoisomerase II this drug would form topoisomerase II cleavable complexes which would be revealed as double strand breaks in

Table 3.2 *Inhibitory concentrations of mAMSA, camptothecin and amiloride on isolated DNA topoisomerase activity as determined by other workers.*

Inhibitor	concentration (μg/ml)	reference
topoisomerase II		
mAMSA	0.1	Rowe *et al.* (1986)
	0.4	Pommier *et al.* (1987)
	0.8	Estey *et al.* (1987)
amiloride	10.0	Figgitt *et al.* (1987)
	46.0	Besterman *et al.* (1989)
topoisomerase I		
camptothecin	0.14	Hertzberg *et al.* (1989)

the form of linear DNA. It is not clear why mAMSA did not appear to inhibit MCF-7 topoisomerase I activity when the reaction was stopped with SDS alone [see photograph 3.6].

Doxorubicin [0.1uM] produced a similar level of inhibition of DNA relaxing activity when the reaction was stopped with either SDS or SDS and proteinase K (see photograph 3.6), resulting in the formation of open circular DNA. This is indicative of topoisomerase I-DNA cleavable complexes being formed in the presence of drug which are converted to single strand breaks in the presence of protein denaturants. It would therefore appear that both methods used to stop the reaction are denaturing the topoisomerase I cleavable complex to reveal single strand breaks [nicked open circular DNA]. Doxorubicin also inhibited topomer formation in a concentration dependent manner, with a parallel increase in open circular DNA formation [see photograph 3.7]. This effect was also independent of which the way in which the reaction was terminated. These results further indicate that topoisomerase I activity is responsible for the relaxing activity of the MCF-7 nuclear extract. Doxorubicin [1 and 5uM, photographs 3.4 and 3.7] totally inhibited topoisomerase I activity of MCF-7 nuclear extract without formation of the single strand breakage associated with cleavable complex formation. This is similar to the inhibition of topoisomerase II DNA cleavage activity of doxorubicin at high concentrations shown by Tewey *et al.* (1984). Previously, doxorubicin has been shown to inhibit topoisomerase II activity with production of a cleavable complex and resultant double strand breaks (Tewey *et al.*, 1984). The absence of double strand break production [formation of linear DNA] in this study would therefore indicate an absence of topoisomerase II in this nuclear extract from MCF-7 cells. The results of this study therefore indicate a role for doxorubicin in inhibition of MCF-7 topoisomerase I, formation of topoisomerase I cleavable complex and subsequent single strand break formation. This could be a contributory factor to the DNA protein associated single and double strand breaks produced by doxorubicin in cellular systems [see section 1.16.3 and 1.16.4.3]. The results described in Chapter 3 of the present study [section 3.3.4.1] show that doxorubicin produced a concentration dependent strand breakage of DNA in MCF-7 cells which is likely to consist of both protein associated and

frank strand breaks. Furthermore, the concentration of doxorubicin [0.05uM] shown to inhibit DNA relaxing activity of MCF-7 nuclear extract in the present study are of similar magnitude to those that produce DNA strand breakage and a cytotoxic response in the MCF-7 cell line *in vitro* [see section 3.3.4.1]. Doxorubicin has also been found to cause protein associated single and double strand breaks in mouse leukaemia L1210 cells (Zwelling *et al.*, 1981), rat mammary tumour cells (Evans *et al.*, 1986), human lung fibroblasts and V79 chinese hamster cells (Lawrence *et al.*, 1988), human leukaemic cells (McGrath *et al.*, 1989) and human colon adenocarcinoma cells (Broggini *et al.*, 1989). A role for topoisomerase II in tumour cell resistance and hypersensitivity to doxorubicin has also been reported [see section 1.16.3].

This section has shown that the supercoiled plasmid DNA relaxing activity displayed by MCF-7 nuclear extract is likely to be topoisomerase I activity. The absence of topoisomerase II activity appears to be related to either a naturally low level of enzyme in MCF-7 cells or due to the growth state of the cells at the time of harvesting. MCF-7 topoisomerase I activity was inhibited by doxorubicin, suggesting a possible role for this enzyme in protein associated DNA strand breakage by doxorubicin in MCF-7 cells.

3.4.3.2 *The effect of CI941, mitozantrone and alkylaminoanthraquinones on topoisomerase activity in MCF-7 nuclear extract*

These studies were extended to investigate the effect of other quinone containing antitumour drugs on topoisomerase I activity in MCF-7 nuclear extract, namely mitozantrone, CI941 and the alkylaminoanthraquinones. At a drug concentration of 1uM all these agents inhibited topomer formation to varying extents with a corresponding formation of open circular DNA [see photograph 3.8]. The AQ's produced an increase in open circular DNA relative to controls when the reaction was stopped with both SDS or SDS and proteinase K. This increase in open circular DNA is probably due to cleavable complex mediated single strand breakage of DNA to produce nicked open circular DNA. This is indicative of

topoisomerase I cleavable complex formation by the AQ's. Mitozantrone and CI941 were the most potent inhibitors, preventing any topoisomerase I mediated relaxation of supercoiled DNA. This would indicate a total inhibition of the enzyme prior to single strand break production (due to drug induced cleavable complex formation) as was found for doxorubicin at high concentrations [see section 3.4.3.1]. Mitozantrone has previously been reported to be a topoisomerase II inhibitor *in vitro*, producing protein associated single and double strand breaks (Ho *et al.*, 1987; Epstein *et al.*, 1986; Heinemann *et al.*, 1988; Locher and Meyn, 1983). Crespi *et al.* (1986) [using MCF-7 cells] and Tewey *et al.* (1984) showed that mitozantrone inhibits mammalian topoisomerase II activity. However, Crespi *et al.* (1986) showed that mitozantrone had no effect on topoisomerase I activity isolated from MCF-7 cells, although the highest concentration tried by these workers was only 0.18uM and a higher concentration of substrate pBR322 DNA was used compared to the present study. Furthermore, the protein concentration of the extract used by these workers is not stated. Protein (topoisomerase) associated single and double strand breaks have been shown to be produced by mitozantrone in L1210 cells (Cohen *et al.*, 1980) and Chinese hamster ovary cells (Locher and Meyn, 1983). From the results of this study a role for topoisomerase I cleavable complex formation could be involved in the mitozantrone mediated DNA strand breakage observed. This is also likely for CI941 as this compound was also shown to generate DNA strand breakage in MCF-7 cells in the present study [section 3.3.4.2]. The alkylaminoanthraquinones inhibited MCF-7 nuclear extract DNA relaxing activity in the following order of potency: 1,4AQ = 1,5AQ = 1,8AQ > 1AQ. However, the AQ's were less potent inhibitors than mitozantrone and CI941. As described above these agents also formed topoisomerase I cleavable complexes as indicated by the increased amount of open circular in drug treated samples relative to the control treated with nuclear extract alone [see photograph 3.8]. 1,4AQ was previously shown by Locher and Meyn (1983) to produce DNA protein associated strand breaks in Chinese hamster ovary cells to a lesser degree than mitozantrone. This correlates with the lower inhibition of topoisomerase activity produced by 1,4AQ relative to mitozantrone shown in the present study. The results of the present study indicate that promotion of MCF-7 topoisomerase I cleavable complex formation may contribute to the total DNA strand breakage produced by AQ's in MCF-7

cells [section 3.3.4.3].

Inhibition of topoisomerase I by quinone antitumour agents could have important implications in the mechanism of cytotoxicity of these agents in the MCF-7 cell line. An involvement of topoisomerase I has been implicated in DNA replication, RNA transcription, chromosome condensation and decondensation and genetic recombination (reviewed by Liu, 1983). The topoisomerase I inhibitor camptothecin inhibits DNA and RNA synthesis (reviewed by Bodley and Liu, 1988). Therefore, by analogy, inhibition of topoisomerase I, as indicated by the present study, could be implicated in the inhibition of DNA and RNA synthesis produced by doxorubicin (reviewed by Gianni *et al.*, 1983), mitozantrone and 1,4AQ (Nishio and Uyeki, 1983) and CI941 (Showalter *et al.*, 1986) and the condensation of nucleic acids by mitozantrone and 1,4AQ (Kapuscinski and Darzynkiewicz, 1984 and 1986).

The mechanism of quinone antitumour agent inhibition of topoisomerase I activity isolated from MCF-7 cells is unknown. However it is likely to be related to the DNA binding affinity of quinones. Until recently inhibitors of topoisomerase were not categorised as DNA binders. However, those 'non-binding' inhibitors such as the epipodophyllotoxin VM-26 and the anthracycline AD32 have recently been shown to bind to DNA (Chow *et al.*, 1988). Thus DNA binding appears to be a prerequisite for inhibition of topoisomerase activity. The order of potency for inhibition of topoisomerase activity in the present study [derived from photograph 3.8] is mitozantrone = CI941 > 1,5AQ = 1,4AQ = 1,8AQ > 1AQ. This correlates with the order of DNA binding affinity of these agents [see table 3.3]. Furthermore, from table 3.3 it can be see that the DNA dissociation rate constants of the AQ's also correlates with their potency to inhibit MCF-7 topoisomerase I activity. The weakest inhibitor of topoisomerase I, 1AQ, having the highest DNA dissociation constant.

There are several possibilities as to how inhibition of topoisomerase I occurs due to DNA binding by these agents:-

Table 3.3 *Binding of quinone antitumour agents with DNA.*

Compound	DNA affinity constant K_{app} M^{-1}	DNA dissociation constant sec^{-1}
doxorubicin	**6.5x10^6	1.84
mitozantrone	*2.6x10^8	nd
CI941	*1.0x10^8	nd
1AQ	1.03x10^6	127
1,4AQ	2.83x10^6	3.82
1,5AQ	3.2x10^6	1.39
1,8AQ	2.35x10^6	1.84

nd=not determined. derived from Gandecha (1985), except **Capranico et al (1989), *Hartley et al (1988).

1. The intercalation of the quinone [reviewed by Neidle and Abraham, 1987] may cause torsional stress within the DNA duplex which induces topoisomerase I to form the cleavable complex. This would be assisted by the intercalation of drug preventing the enzyme from forming its normal interaction with DNA at a particular site. This view is supported by the inhibition of cleavable complex formation at high concentrations of doxorubicin, mitozantrone and CI941 [see photographs 3.6 and 3.8]. This could be due to drug intercalation causing unwinding of the DNA so that topoisomerase I cannot bind.

2. Intercalation may enable the quinones to specifically interact with topoisomerases therefore stabilising the cleavable complex. This possibly occurs through an interaction between the parts of the drug molecule which protrude from the intercalation site and the DNA-enzyme complex. For example the hydroxyethylethylamino side chains of mitozantrone, CI941 and the AQ's, and the aminosugar of doxorubicin [see figures 1.32, 1.33 and 1.35]. This is supported by the potency of a series of acridine topoisomerase II inhibitors being related to the 'bulkiness' of their side chains (Pommier *et al.*, 1987).

3. The quinones may be able to interact directly with topoisomerase I.

These possibilities are somewhat complicated by the fact that different drugs induce topoisomerase II associated strand breaks at different DNA sites and the general lack of correlation between protein associated strand breaks and cytotoxicity of different drug classes (Rowe *et al.*, 1986). Furthermore an understanding is required of whether cleavable complex formation is important in cell kill *per se* or whether it is the inhibition of topoisomerase function which is important. In addition, there is no clear understanding on how cleavable complexes are converted to lethal DNA strand breaks *in vivo*. Avemann *et al.* (1988) showed that camptothecin induced topoisomerase I mediated strand breakage at the replication forks of SV40 minichromosomes. Also, Schneider *et al.* (1989) found that mAMSA was maximally cytotoxic in cells undergoing RNA and protein synthesis. When RNA and protein synthesis were inhibited mAMSA produced no change in the level of cleavable complexes formed,

suggesting that an active event is required for conversion of complex formation to cell kill (Schneider *et al.*, 1989).

3.4.4 *The effect of quinone antitumour agents on cellular DNA.*

Cellular DNA strand breakage was determined in MCF-7 human breast cancer cells after treatment with quinone containing antitumour agents. This was determined in order to relate the propensity for redox cycling and the cytotoxic potential of these agents to their ability to produce cellular DNA damage.

3.4.4.1 *The effect of doxorubicin, mitozantrone and CI941 on DNA strand breakage in MCF-7 cells.*

Doxorubicin produced DNA strand breakage in MCF-7 cells in a drug concentration dependent manner [figure 3.12A]. Table 3.4 relates the strand scission factors observed for doxorubicin in MCF-7 cells with the cell kill produced by doxorubicin following a one hour exposure and different periods of further drug-free incubation period [derived from figure 3.12B]. It can be seen from this table that as the post-exposure incubation time increases the cell kill observed for a given level of DNA strand scission also increases. The low level of strand breakage observed for 0.1 and 1.0uM doxorubicin correlated with almost no cell kill following a 2.5 or 6 day post-exposure incubation period. However, following an 11 day post-exposure incubation both these concentrations produced cell kill. This indicates that there is a time delay before the DNA strand breakage observed is expressed as cell kill. This suggests that although low doxorubicin concentrations produced only a low level of DNA strand scission, sufficient numbers of these DNA lesions are non-repairable and ultimately lethal to the cell. The results of the present study are supported by the work of others who also measured doxorubicin strand breakage under deproteinised conditions. Doxorubicin has been found to produce strand breakage in other human tumour cell lines including human myeloma cells (Bellamy *et*

Table 3.4 *Relationship between doxorubicin mediated DNA strand breakage and cytotoxicity in MCF-7 cells.*

doxorubicin conc [μM]	Strand scission factor*	% Cell kill A	B	C
0.1	0.044	10.1 + 13.0	14.4 + 19.7	58.1 + 12.9
1.0	0.041	23.9 + 6.5	30.0 + 28.4	85.9 + 1.9
10.0	0.095	58.0 + 5.1	71.9 + 6.0	88.4 + 10.0
100.0	0.153	nd	78.6*	84.0 + 0.6

A = 1hour exposure + 60 hours drug-free growth
B = 1hour exposure + 6 days drug-free growth
C = 1hour exposure + 11 days drug-free growth

values are mean + sd (except*)
nd = not determined

al., 1988), CCRF- CEM human leukaemia cells (McGrath *et al.*, 1989), A549 human lung adenocarcinoma cells (Lawrence, 1988) and also in other mammalian cell lines such as P388 leukaemia cells (Capranico *et al.*, 1989; Deffie *et al.*, 1988) and L1210 mouse leukaemia cells (Potmesil *et al.*, 1988). Many of the above studies do not discriminate between protein associated and non-protein associated (frank) strand breakage. Doxorubicin has also been shown to produce frank DNA strand breakage in Chinese hamster V79 cells (Iliakis and Lazar, 1987), human uterine sarcoma cells (Scudder *et al.*, 1988), L1210 mouse leukaemia cells (Potmesil *et al* 1983 and 1984; Ross and Smith, 1982), P388 lymphocytic tumours (Goldenberg *et al.*, 1986) and human colon carcinoma cells (Johnston *et al.*, 1983). The DNA strand breakage observed for doxorubicin in the present study is likely to represent both protein associated and frank strand breakage. Doxorubicin strand breakage *in vitro* revealed by proteinase K have been shown to be due to topoisomerase II cleavable complex formation (Zwelling *et al.*, 1981)[see section 1.16.3]. The results of the present study have also shown doxorubicin to be an inhibitor of MCF-7 topoisomerase I activity with formation of topoisomerase I cleavable complexes (see section 3.3.3). SDS and/or proteinase K treatment of these cleavable complexes result in the formation of single strand breaks. Therefore, the inhibitory action of doxorubicin on topoisomerase I could contribute to the strand breakage observed for this agent in MCF-7 cells. Frank DNA strand breakage produced by doxorubicin in MCF-7 cells is likely to be due to the redox cycling activity of this agent observed in the present study (see chapter 2) which resulted in formation of hydroxyl radicals [section 2.3.1.6]. This is supported by the work of Potmesil *et al.* (1984) who showed that doxorubicin produced frank DNA strand breakage in mouse leukaemic L1210 cells at concentrations > 2.8uM which were reduced in number by the oxygen free radical scavengers SOD, catalase, DMSO and ethanol. Furthermore, frank strand breakage was not observed under hypoxic conditions. The mechanism of hydroxyl radical attack on DNA is fully discussed in section 1.9.2.1 and illustrated in figures 3.19-3.22. Mitozantrone and CI941 [100uM] produced a similar level of DNA strand breakage to doxorubicin [table 3.5]. At an LD50 concentration, mitozantrone ($5x10^{-9}$M) produced a similar amount of strand scission to that produced by a concentration of 100uM (see figure 3.14). This indicates that mitozantrone mediated strand breakage is saturable at

Figure 3.5 *Strand scission factors for doxorubicin, mitozantrone, CI941 and alkylaminoanthraquinones in MCF-7 cells.*

Drug	Concentration ED50	100uM
doxorubicin	0.067*	0.153*
mitozantrone	0.211 + 0.07	0.127 + 0.28
CI941	0.0612 + 0.03	0.179 + 0.24
1AQ	0.129 + 0.06	0.436 + 0.17
1,4AQ	0.118 + 0.08	0.094*
1,5AQ	0.285 + 0.09	0.652 + 0.18
1,8AQ	0.129 + 0.04	0.534 + 0.08

Values are mean + sd of three determinations [except *(2)]

ED50 concentrations [M] were mitozantrone [$5x10^{-9}$], CI941 [$1.5x10^{-10}$], doxorubicin [$3.0x10^{-6}$], 1AQ [$6x10^{-8}$], 1,4AQ [$1.2x10^{-6}$], 1,5AQ [$12.3x10^{-6}$] and 1,8AQ [$0.5x10^{-6}$].

high drug concentrations. CI941 ($2x10^{-10}$M) produced 60% less strand breakage than at 100uM (figure 3.14). At an LD50 concentration ($3.0x10^{-6}$M) doxorubicin produced a similar level of strand breakage to CI941, but less than mitozantrone (see table 3.5). These results indicate that mitozantrone is more potent than CI941 at generating strand breakage in MCF-7 cells. However, at LD50 concentrations both mitozantrone and CI941 are considerable more potent than doxorubicin at producing strand breakage. This correlates with the increased cytotoxic potency of mitozantrone and CI941 relative to doxorubicin [table 4.5], indicating that DNA strand breakage is involved in the cell kill of these agents. Furthermore, mitozantrone was also been shown to be more efficient than doxorubicin at producing DNA strand breakage in T47D human breast cancer cells [Epstein *et al.*, 1988]. The relative potency of DNA strand breakage formation by doxorubicin, mitozantrone and CI941 correlates with their DNA binding affinities [table 3.3]: mitozantrone > CI941 > doxorubicin. The DNA binding constants of mitozantrone and CI941 has previously been correlated in the present study with their enhanced abilty to inhibit MCF-7 topoisomerase I activity [section 3.4.3.2]. Furthermore, mitozantrone has previously been shown to inhibit MCF-7 topoisomerase II activity (Crespi *et al.* 1986) and produce protein associated strand breaks *in vitro* (Heinemann *et al.*, 1988; Epstein *et al.*, 1988). Therefore, it is likely that cellular DNA strand breakage by mitozantrone and CI941 in the present study are due to proteinase K treatment (part of the alkaline elution technique) revealing topoisomerase I and II cleavable complex mediated single and double strand breaks. The saturable nature of mitozantrone mediated DNA strand breakage in MCF-7 cells would also support this concept of an enzyme [topoisomerase] being involved. Moreover, mitozantrone and CI941 were found not to redox cycle in MCF-7 cells [2.4.2] in the present study, hence these agents are unlikely to produce hydroxyl radical mediated DNA strand breaks. The high DNA binding constants of mitozantrone and CI941 are likely to result in these agents accumulating in the nucleus, resulting in a persistance of the DNA lesions produced. This could account for the much increased cytotoxic potency of these agents relative to the other compounds in this study [see tables 4.5 and 4.6]. Other workers have found that mitozantrone induced strand breaks persist after removal of drug, including Ho *et al.* (1987) and Locher and Meyn (1983). As discussed in section 3.4.3 proteinase K revealed

single and double DNA strand breaks do not exist as strand breaks *in vitro* because they are masked by the presence of the topoisomerase-DNA complex. It is likely that an active process such as DNA transcription or replication needs to occur before topoisomerase cleavable complexes are converted into lethal DNA lesions.

3.4.4.2 *The effect of alkylaminoanthraquinones on DNA strand breakage in MCF-7 cells*

At a concentration of 100uM 1AQ, 1,5AQ and 1,8AQ produced a large amount of strand breakage [see figure 3.17]. In contrast, 1,4AQ (100uM) produced only a small amount of strand breakage, similar in quantity to that produced by doxorubicin, mitozantrone and CI941 at this concentration [see table 3.5]. The increased amount of strand scission by 1AQ, 1,5AQ and 1,8AQ at 100uM compared to 1,4AQ, mitozantrone and CI941 correlates with the increased propensity of these AQ's to redox cycle and form hydroxyl radicals in MCF-7 cells (see section 2.4.3). A role for hydroxyl radicals in the cellular DNA strand breakage produced by these agents is supported by the similar elution profile of MCF-7 cellular DNA from cells treated with gamma radiation (appendix figure A13). Radiation has been proposed to mediate cellular DNA damage by hydroxyl radical formation (reviewed by Ward, 1987). The mechanism of hydroxyl radical mediated DNA strand breakage is discussed in sections 1.9.2 and 3.4.1.1 and illustrated in figures 3.19-3.21. In contrast, 1,4AQ, mitozantrone and CI941 have been shown not to redox cycle in MCF-7 cells [sections 2.4.3 and 2.4.2]. At the LD50 concentrations of 1AQ (4.0uM), 1,5AQ (12.3uM) and 1,8AQ (0.5uM) a decrease in DNA strand scission was observed compared to higher (100uM) concentrations of these agents, whereas, at 1.2uM (LD50 concentration) and 100uM, a similar level of strand breakage was observed for 1,4AQ [table 3.5]. The AQ's produced a similar level of strand scission to doxorubicin, mitozantrone and CI941 at LD50 concentrations (table 3.5). However, the LD50 concentrations of mitozantrone and CI941 were considerably lower than those for doxorubicin or AQ's. The lower level of strand breakage by 1AQ, 1,5AQ and 1,8AQ at LD50 concentrations correlates with a lower level of hydroxyl radical formation at these concentrations. Similar to

mitozantrone and CI941, the AQ's have also been shown to be inhibitors of topoisomerase I activity in MCF-7 cells in the present study [section 3.4.3.2]. It is therefore likely that DNA strand breakage by 1,4AQ is due to topoisomerase I or II cleavable complex formation. However, these compounds had reduced potency to inhibit this enzyme compared to mitozantrone and CI941 [see photograph 3.8]. It is likely that proteinase K revealed topoisomerase I or II cleavable complex mediated single and double strand breaks contribute to the DNA strand breakage observed for the AQ's in MCF-7 cells. 1,4AQ has been previously shown to produce protein associated strand breaks in Chinese hamster ovary cells, with 250x less efficiency than mitozantrone (Locher and Meyn, 1983). Furthermore, 1,4AQ induced strand breaks were repaired more efficiently than those produced by mitozantrone [Locher and Meyn, 1983]. The higher efficiency of strand break formation by mitozantrone and CI941 in the present study, compared to AQ's, also correlates with the higher DNA binding constants of these compounds compared to the AQ's [see table 3.3].

The relationship between DNA strand breakage, redox cycling, inhibition of topoisomerase and cytotoxicity of the compounds in the present study will be further discussed in chapter 5.

CHAPTER 4

An investigation of cytotoxicity and uptake of quinone containing antitumour agents in MCF-7 human breast cancer cells

CHAPTER 4

An investigation of cytotoxicity and uptake of quinone containing antitumour agents in MCF-7 human breast cancer cells

4.1 Introduction

The previous chapters have investigated the propensity of the quinone antitumour agents in this study to redox cycle [chapter 2] and produce DNA strand breakage [chapter 3] in the MCF-7 cell line. In this chapter the cytotoxicity of these agents is investigated in order to relate cytotoxic potential to the propensity of these agents to redox cycle and generate DNA strand breakage in MCF-7 cells.

4.2 Methods

4.2.1 *Determination of cytotoxicity in MCF-7 cells*

Two methods were used to determine the cytotoxicity of quinone antitumour agents in MCF-7 cells. The first method involved determining the number of surviving cells after a 24 hour drug exposure. This method allowed determination of drug short term cytotoxicity. The second method involved exposing the cells to drug for one hour then growing on the cells for various time periods. This enabled the effect of the drug on the clonogenic potential of the cells to be determined.

Method 1:- Firstly, several flasks of MCF-7 cells were grown up, harvested and resuspended in growth medium. The cells were dispersed by passing the suspension through a 25 gauge followed by a 21 gauge needle. The cells were counted using a Coulter counter and the cell concentration adjusted to $10^5 ml^{-1}$ in growth medium and aliquots of cell suspension (1ml) pipetted into a 12 well plate. At this point the well plates were incubated in a sealed plastic bag in an atmosphere of 5% CO_2/95% air for 24 hours at 37C. After 24 hours the medium was poured off the cells and replaced with medium containing appropriate serial dilutions of a filtered [1.0u acrodisc, Flow Labs] drug solution or growth medium in the case of controls. The well plates were then reincubated for 24 hours at 37C. After 24 hours the drug solutions were poured off and the wells washed twice using ice cold Dulbecco's phosphate buffered saline. EDTA, (0.1%, 0.5ml) was added to each well for 30 seconds and then poured off and the plates incubated for 15 minutes at 37C. The cells were resuspended in 1ml of DPBS and dispersed by passage through a 25 gauge followed by a 21 gauge needle (several times). The cell suspension in each well was transfered to isotonic saline (20ml) by means of a syringe and the cell concentration determined using a Coulter counter. All results were related to untreated control cell counts.

Method 2:- This method is essentially the same as method 1 except for

the following details:-The passaged MCF-7 cells were adjusted to a concentration of $10^4$to $10^5 ml^{-1}$ pipetted into 12 well plates in aliquots [1ml]. After 24 hours incubation the medium was replaced with drug dilution or growth medium alone and incubated at 37C for 1 hour. After one hour the drug solutions were removed and the plates washed with ice cold DPBS (1ml) for one minute following which fresh growth medium (1ml) was placed in each well. The plates were incubated for various time periods [96hrs-17days] at 37C with a media change every five days of incubation. The cell numbers were determined as method 1.

In order to determine the effect of oxygen free radical scavengers on MCF-7 cell survival in the presence of quinone antitumour agents method 2 was modified as follows:-

Prior to addition of drug the cells were incubated (37C) for thirty minutes in in serum supplemented RPMI-1640 (0.5ml) containing double the required final concentration of scavenger. Following this period more growth medium was added (0.5ml) containing double the required concentration of drug. Following one hour further incubation, the drug/scavenger containing medium was removed, the monolayer washed with ice cold PBS, fresh medium added and cell survival assessed following a further six day incubation as described above.

4.2.2 *Determination of quinone antitumour agent uptake into MCF-7 cells*

4.2.2.1 *Using high performance liquid chromatography (HPLC)*

Drug uptake into MCF-7 cells was determined by measuring drug in the medium (by HPLC) after incubation of cells with drug. Incubations were carried out as follows. Firstly log phase MCF-7 cells were harvested and counted as described in appendix A1. The cells were suspended in RPMI-1640 growth medium supplemented with 10% foetal bovine serum (FBS)

at a concentration of 10^6 cells ml^{-1}. The cells were dispersed by passage through a needle [as described in section 4.2.1] and centrifuged [900rpm, 10 minutes, 4C] using a refrigerated bench centrifuge [CR422, Jouan] to pellet the cells. RPMI-1640 medium supplemented with 10% FBS was placed in a glass conical flask [20ml] which had been treated with trichloromethylsilane to prevent binding of drug or adhesion of cells. An appropriate volume of drug was added to the medium to give a final concentration of 15uM. Three samples (200ul) were removed from the flask and transferred into eppendorf tubes (1.5ml) and used as controls. The drug-containing medium was pre-warmed to 37C in a water bath. To initiate the uptake study the cell pellet was resuspended in the drug-containing medium [to give a cell concentration of 10^6 cells ml^{-1}] and transfered to the incubation flask. Three samples (200ul) were immediately removed from the cell suspension and placed in eppendorf tubes. Ice cold ethanol [50ul] was added to the tubes [to precipitate serum protein] and the tubes spun for 2 seconds in a microcentrifuge [Hema-C, Jouan]. The supernatants were removed and placed in HPLC sample tubes [08-CPVIA, Chromacol]. The cell pellet was then resuspended in ice cold PBS [150ul] and the tubes spun for 2 seconds in a microcentrifuge. The supernatants were again removed and pooled with those of the first centrifugation step in the HPLC sample tubes. Finally the tubes were capped and sealed ready for HPLC autoinjection. The control samples were treated in an identical manner to above. Further triplicate samples of cell suspension were removed from the incubation flask at 5, 15, 30, 60 and 90 minutes and treated as above.

The drug remaining in the supernatant was quantitated using a Phillips HPLC [PU 4100 Liquid Chromatograph, PU 4110 uv/vis Detector (254nm), PU4700 autoinjector and Walters computer system JCL6000 with Chromat data capture system (Jones Chromat, 1988). The solvent system used consisted of CH_3CN [28%] and ammonium formate (80mM, pH 3.25) [72%]. For each anthraquinone studied a calibration curve was constructed using drug standards (5-15uM) prepared in RPMI-1640 medium supplemented with FBS [10%]. The drug concentration in each sample was calculated from the calibration curve [Maine, J, personal communication] and the results expressed as nmol of drug taken up per 10^6 cells.

4.2.2.2 *Using polarography*

Polarography is an electrochemical method in which the changes of current, resulting from the electrolysis of the solution under investigation are followed using a mercury electrode and a gradually applied voltage (reviewed by Bersier, 1983; Heyrovsky, 1968; Zuman, 1964). Mitozantrone can undergo oxidation and reduction and can therefore be detected polarographically under oxidative or reductive conditions. This method was used to determine mitozantrone uptake into MCF-7 cells. Polarography was carried out using a 626 Polarecord combined with a 663VA stand [Metrohm]. The optimal protocol for determination of mitozantrone involved utilisation of a Stelte type micro cell with differential pulse polarography (50Mv pulse) and a hanging mercury drop electrode [HMDE] under reductive conditions. The following methods describe experiments which were carried out to characterise the polarographic determination of mitozantrone with a view to using this technique for determining cellular uptake of this drug.

4.2.2.2a *Determination of mitozantrone (0.5uM) polarographically*

The optimal settings for the polarograph were found to be hanging mercury drop electrode mode, differential pulse 50mV, drop size 1, scan speed 5, measurement period 2 seconds, voltage sweep -0.0 to -1.4V, damping 1 and sensitivity 0.1nA/mm.

Initially a background voltage sweep was carried out using Dulbecco's phosphate buffered saline [DPBS] (0.1M, pH 7.4) after deaerating for 10 minutes with a steady stream of nitrogen [oxygen free (BOC)]. Several voltage sweeps were then carried out on mitozantrone solution (0.5uM, in DPBS) after deaerating for 3-5 minutes.

4.2.2.2b *Preparation of a calibration curve for mitozantrone (0-10uM) using polarography*

A range of mitozantrone concentrations made up in DPBS were determined in triplicate using the polarographic parameters described in section 4.2.2.2a. The height of the reduction peaks at -0.95 and -1.04V were measured for each drug concentration. Appendix figure A9 shows a typical calibration curve.

4.2.2.2c *Polarographic determination of mitozantrone uptake into MCF-7 cells*

MCF-7 cell suspension ($10^6 ml^{-1}$) in DPBS was placed in a glass conical flask [20ml] pre-coated with trichloromethylsilane [Sigma] and incubated in a water bath at 37C. Cell suspension [2ml] was removed and placed in a centrifuge tube [treated as above] at 37C for use as a drug-free control. A Mitozantrone stock solution (5mM) was added to the flask to give a final concentration of 10uM. After mixing, three samples [1ml] were removed, placed in Eppendorf tubes and spun for 2 seconds using a microcentrifuge. The supernatants were removed, placed in clean Eppendorf tubes, and the cell pellets resuspended in ice cold DPBS [0.5ml] and centrifuged as above. The supernatants of the two centrifugation steps were then pooled. Further triplicate samples of cell suspension were removed at 30, 60, 90, 120, 180, 240 and 300 minutes and treated in an identical fashion. At this stage the supernatants could be stored in a freezer [-20C] for later analysis. Also at regular time intervals, samples of cell suspension [125ul] were removed from the drug containing and drug-free suspensions and the cell viability assessed using trypan blue exclusion [as described in appendix A1.9].

The concentration of mitozantrone in the supernatants was determined polarographically by measuring the peak height of the -0.95V reduction peak. The polarographic parameters employed were as described in section 4.2.2.2a. As a standard, samples of a solution of mitozantrone [10uM] in DPBS were treated in an identical manner to cell suspension,

the -0.95V peak heights of the experimental samples being compared to the mean peak height of the standards and expressed as a percentage of this value. From this the absolute amount (nmoles) of drug taken up by the cells could be calculated. The concentration of mitozantrone taken up by the cells was expressed as the amount of drug [nmoles] taken up per 10^6 cells.

4.3 Results

4.3.1 Cytotoxicity and uptake of quinone antitumour agents in MCF-7 cells

Cytotoxicity was determined by several different protocols as described in section 4.2.1. This enabled comparison of short term and long term cytotoxic effects of these agents as well as the effect of different exposure times on cytotoxicity. In addition, the effect of oxygen radical scavengers on cell survival after treatment with selected drugs was investigated. Figure 4.1 shows that when drug exposure was carried out 24-48 hours after seeding the MCF-7 cells were in an exponential growth phase.

Drug uptake was measured by HPLC [as described in section 4.2.2] in order to determine whether any differences in cytotoxicity observed between drugs could be accounted for by differences in cellular uptake.

4.3.1.1 *Cytotoxicity of doxorubicin in MCF-7 cells*

Cytotoxicity of doxorubicin after a 24 hour exposure followed by immediate determination of the surviving cell fraction was investigated. Figure 4.2A shows the results of two experiments which were carried out. Doxorubicin was cytotoxic to MCF-7 cells between 0.01 and 100uM. The drug concentrations that killed 50% of the cells in two determinations [Lethal Dose 50% or LD50] were 0.17 and 0.38uM. The effect of doxorubicin on MCF-7 cells which were fully confluent was also determined and is shown in figure 4.2B. Exposure time was 24 hours followed by immediate counting of surviving cells. In this system doxorubicin failed to kill more than 40% of the cells.

The cytotoxicity of doxorubicin was determined after cells were further

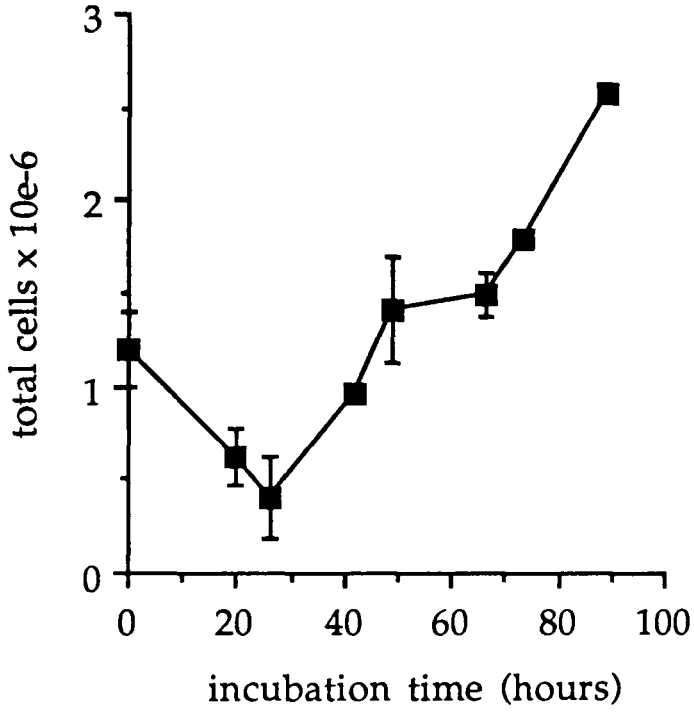


Figure 4.1 *Growth curve for MCF-7 cells*

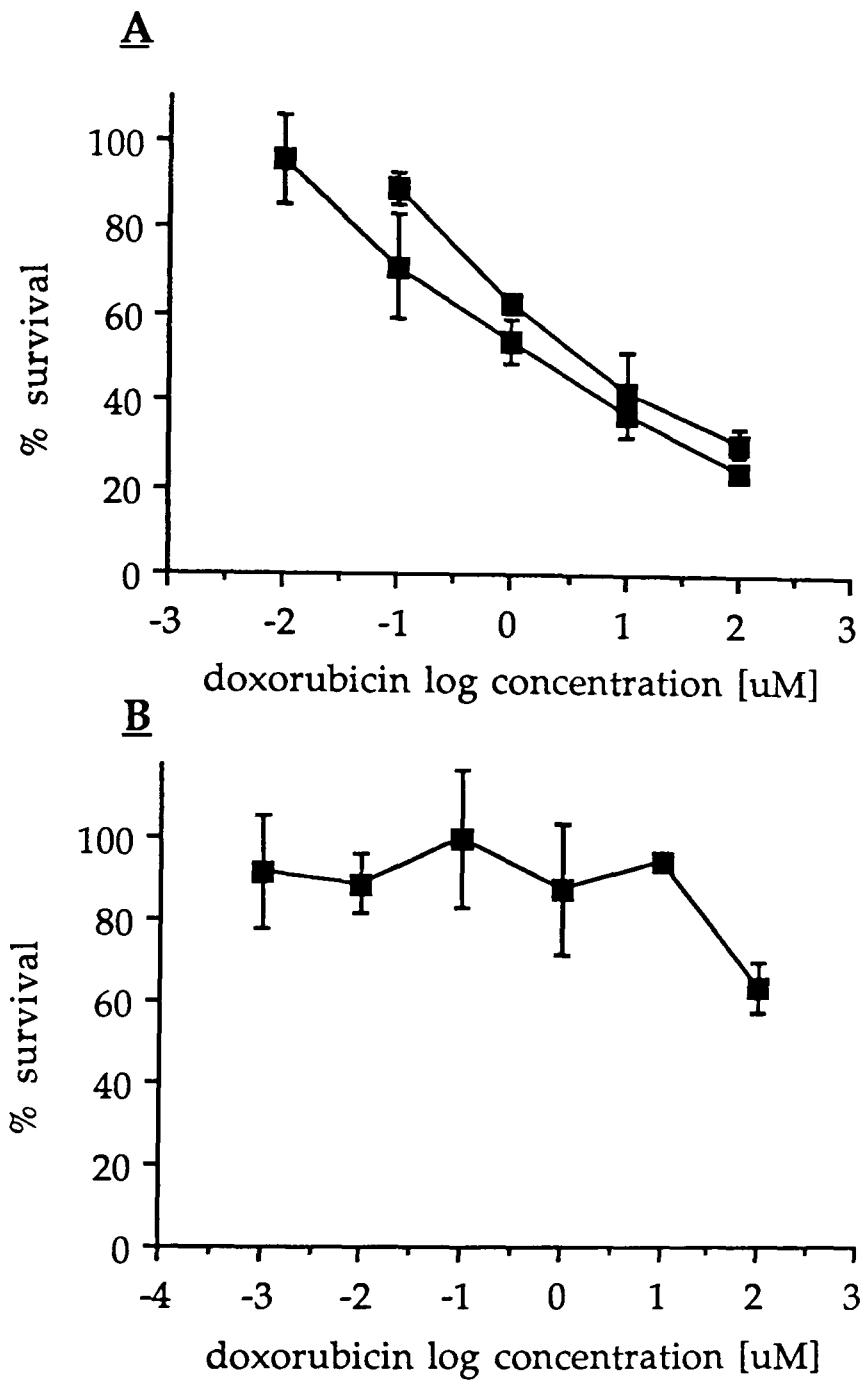


Figure 4.2 *Cytotoxicity of doxorubicin in exponentially growing [A] and quiescent [B] MCF-7 cells after a 24 hour exposure*

10^5 cells were seeded per well and cultured for 24 hours prior to drug exposure. Surviving cells were counted immediately after drug exposure. Final control cell numbers were $2.62 + 0.5 \times 10^5$/ well.
Values are mean + sd of three determinations.

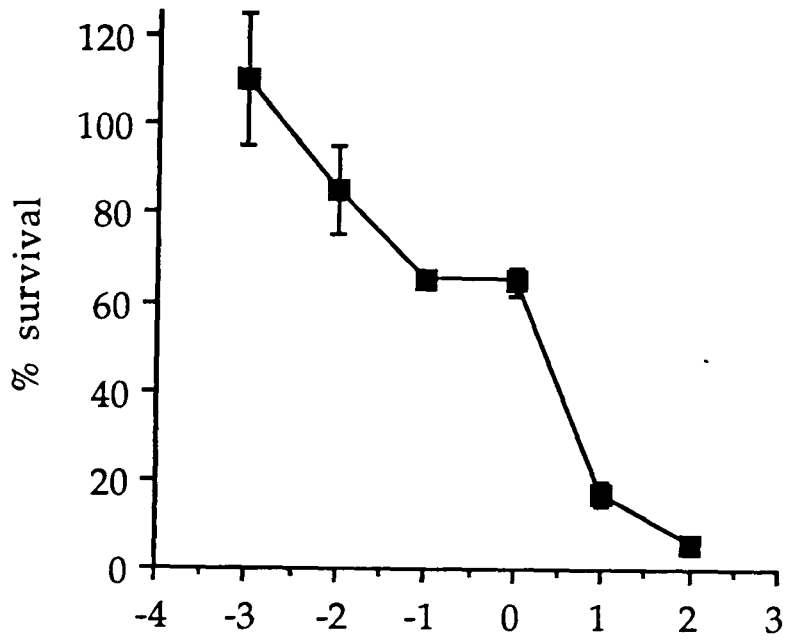


Figure 4.3 *Cytotoxicity of doxorubicin in MCF-7 cells after a 24 hour exposure followed by a further 96 hour drug-free incubation*

$5x10^4$ cells were seeded per well and cultured for 48 hours prior to drug exposure. After a further 96 hour incubation control cell numbers were $2.1 + 0.1x10^5$/well. Values are mean + sd of three determinations.

incubated [96 hours] after exposure to drug for 24 hours [figure 4.3]. The LD50 concentration observed for doxorubicin using this protocol was 0.2uM. Uptake of quinone antitumour agents by MCF-7 cells was found to be maximal after 60 minutes of incubation [see section 4.3.2.1]. Cytotoxicity after a one hour drug exposure period was therefore investigated. In addition the effect of extending the incubation period after drug exposure was investigated. Other workers have used one hour drug exposures with a range of incubation times post-exposure [see table 4.6]. Figure 4.4 shows the effect of exposing MCF-7 cells to doxorubicin for one hour followed by 60 hour or 11 day further drug-free incubation. Appendix figure A8 shows the cytotoxicity of doxorubicin in MCF-7 cells following a one hour exposure and six day further growth period. Table 4.1 summarises the LD50 concentrations obtained using these different protocols. It can be seen from this table that the LD50 concentration was inversely related to post-exposure incubation period.

4.3.1.2 *The effect of oxygen radical scavengers on doxorubicin cytotoxicity in MCF-7 cells*

The effect of oxygen free radical scavengers on the cytotoxicity of doxorubicin [3.0uM (LD50 concentration)] after a one hour exposure and 6 day further drug free incubation is shown in figure 4.5. Cells were incubated with scavenger for 30 minutes prior to addition of drug. Scavenger and drug were incubated for a further hour before removal, washing [with PBS] and addition of fresh medium. DMSO [100mM] and catalase [3000 units ml^{-1}] slightly increased the proportion of surviving cells relative to cells treated with doxorubicin [18.4% and 9.9% respectively]. However, N-acetyl cysteine [100mM] and superoxide dismutase [150ug ml^{-1}] had no protective effect.

In a further study figure 4.6 shows the effect of increasing the scavenger concentration used. Catalase [6000 units ml^{-1}], superoxide dismutase [600ug ml^{-1}] and SOD plus catalase [3000 units/300ug ml^{-1}] increased cell survival by 16.9%, 11.1% and 15.7% respectively. DMSO [200mM] had no effect on cell survival.

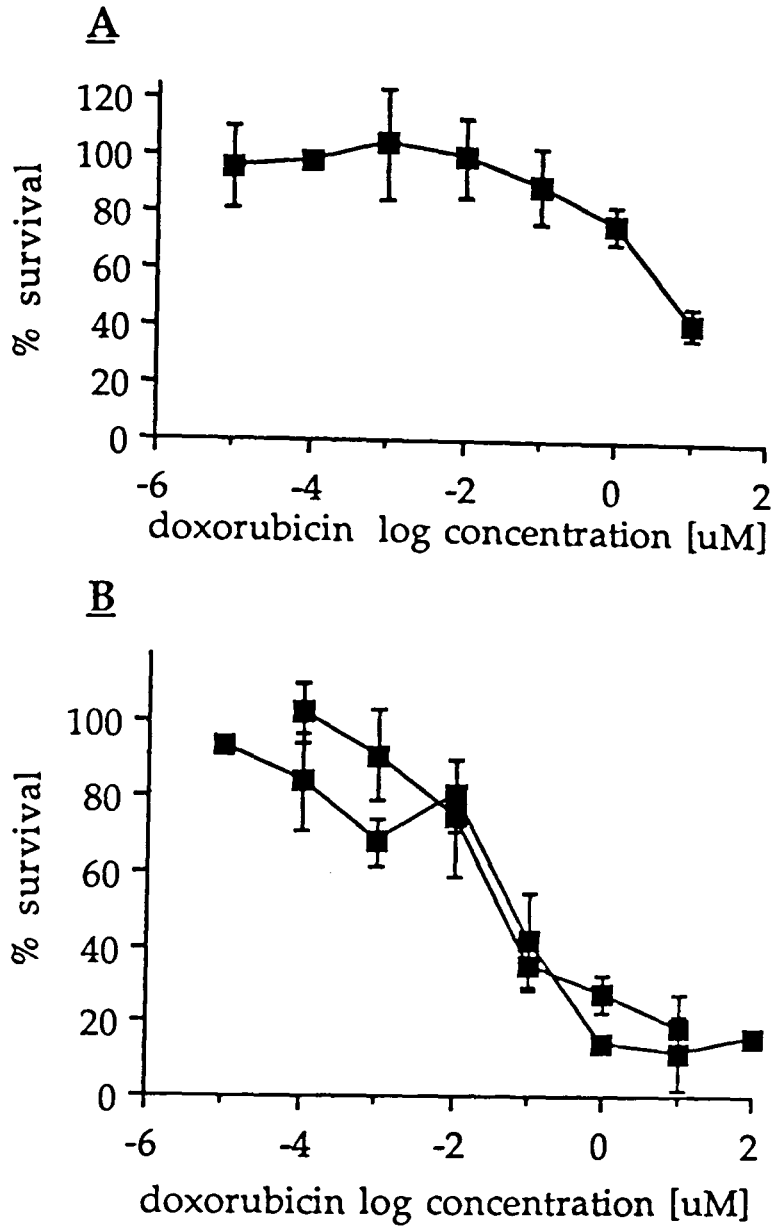


Figure 4.4 *Cytotoxicity of doxorubicin in MCF-7 cells after a one hour exposure followed by a further 60 hour [A] or 11 day [B] drug-free incubation*

A: 10^5 cells were seeded per well and incubated for 24 hours prior to exposure to drug. Final control cell numbers were $1.38 + 0.14 \times 10^5$/well. B: 10^4 cells were seeded per well and incubated for 24 hours prior to exposure to drug. Final control cell numbers were 5×10^5/well. Values are mean + sd of three determinations.

Table 4.1 *Effect of post-exposure incubation time on the LD50 concentration of doxorubicin in MCF-7 cells.*

post exposure incubation time [hours]	LD50 concentration [uM]
60	5.6
144	3.0
264	0.06

Exposure time was one hour. Cells/incubate was 10^5 [60], 10^4 [144], and 10^4 [264]. Data derived from Figure 4.4 and Figure A8

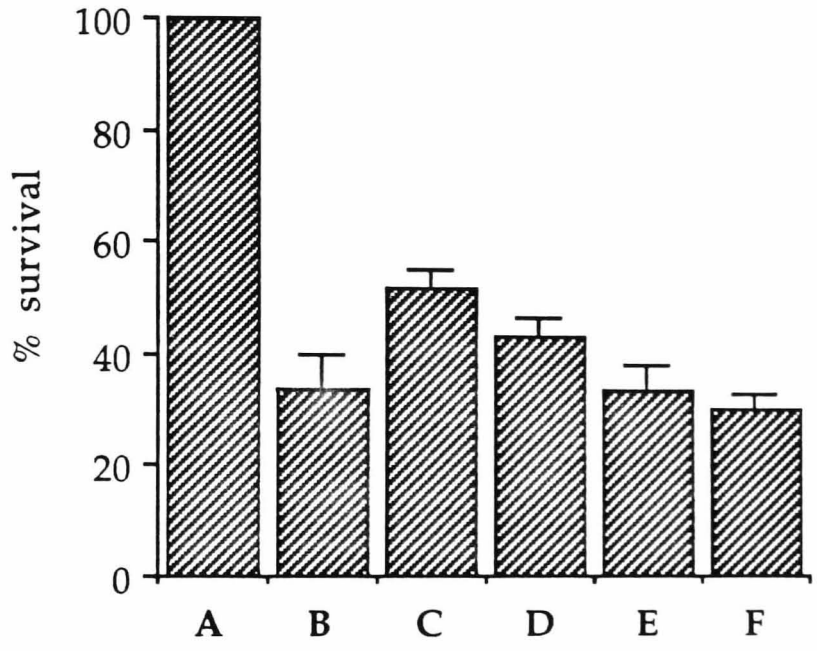


Figure 4.5 *Effect of oxygen free radical scavengers on the cytotoxicity of doxorubicin in MCF-7 cells*

10^4 cells were seeded per well and cultured for 24 hours prior to exposure to drug. Cells were incubated with **A**: no drug, or **B**: doxorubicin [3uM] with **C**: DMSO [100mM], **D**: catalase [3000 units], **E**: SOD [150ug] and **F**: n-acetyl cysteine [100mM]. After 6 days further incubation control cell numbers were $4.0 + 1.15 \times 10^4$/well. Values are mean + sd of three determinations.

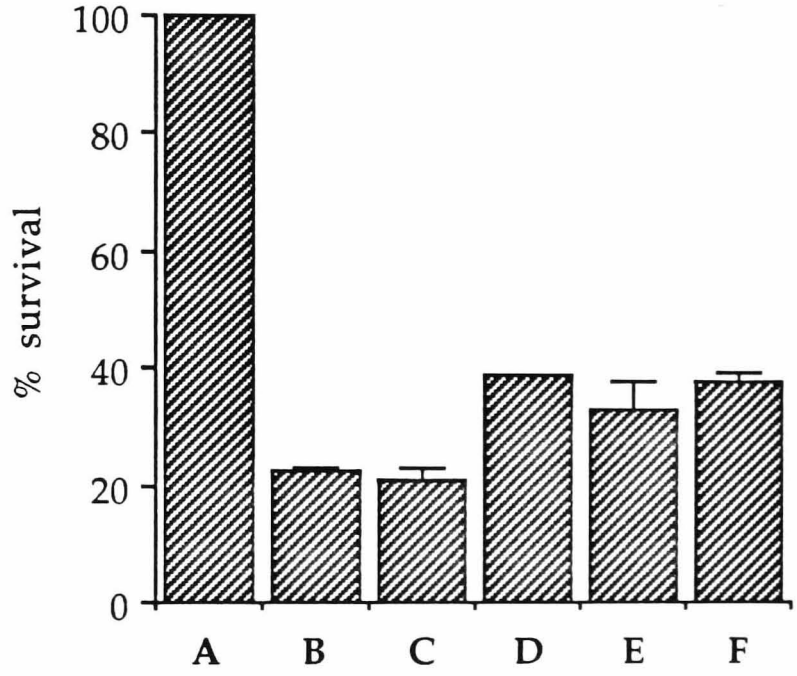


Figure 4.6 *Effect of oxygen free radical scavengers on the cytotoxicity of doxorubicin in MCF-7 cells*

10^5 cells were seeded per well and cultured for 24 hours prior to drug exposure. Cells were incubated for one hour with **A**: no drug, or **B**: doxorubicin [3uM] with **C**: DMSO [200mM], **D**: catalase [6000 units], **E**: SOD [600ug], **F**: catalase and SOD [3000 units/300ug]. After 6 days further incubation the control cell numbers were $8.96 + 0.64 \times 10^4$ Values are mean + sd of three determinations.

4.3.1.3 *Cytotoxicity of mitozantrone and CI941 in MCF-7 cells*

Figure 4.7A (also appendix figure A9) shows the effect of exposing MCF-7 cells to mitozantrone or CI941 for 24 hours followed by immediately counting the surviving cells. The mean LD50 concentrations for mitozantrone and CI941 derived from this data were 2.4uM and 0.8uM respectively.

The effect of incubating cells for a further 96 hours after exposure to drug for 24 hours was also investigated. Figure 4.7B shows the cytotoxicity profile obtained. In this system mitozantrone had an LD50 of $1.62x10^{-10}$M and CI941, a mean LD50 of $4.6x10^{-10}$uM [determined from two independent experiments]. The cytotoxicity range of these drugs was from 10^{-12} to 10^{-8}M.

The effect of exposure of MCF-7 cells to mitozantrone and CI941 for one hour was investigated in a similar manner to doxorubicin. After a 60 hour post-exposure incubation the LD50 concentrations obtained were $1.3x10^{-7}$M for mitozantrone and $1.6x10^{-8}$M for CI941 [figure 4.8A]. The effect of increasing the post-exposure incubation period to 6 days is shown in figure 4.8B. From this study LD50 concentrations of $5.2x10^{-9}$M and $1.5x10^{-10}$M were derived for mitozantrone and CI941 respectively. Thus, increasing the post-exposure incubation period decreased the drug LD50 concentration. Figure 4.9 shows the cytotoxicity of mitozantrone after a one hour exposure and a further 11 or 17 day drug free incubation [with media change on day 5 and 8 respectively]. The LD50 concentrations obtained using these protocols [shown in table 4.2] were inversely related to post-exposure incubation period.

4.3.1.4 *Cytotoxicity of alkylaminoanthraquinones in MCF-7 cells*

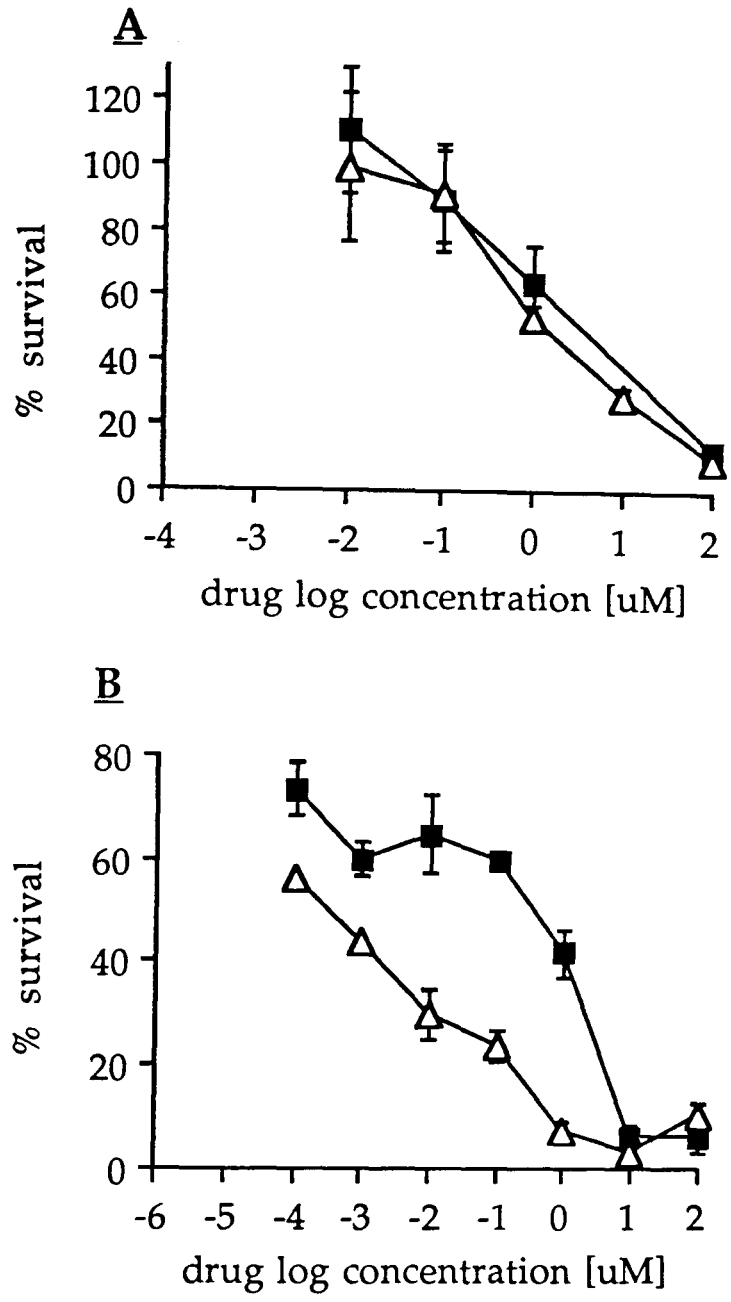


Figure 4.7 *Cytotoxicity of mitozantrone [■] and CI941 [△] in MCF-7 cells after a 24 hour exposure [A] and a 24 hour exposure followed by a 96 hour drug-free incubation period [B].*

A: Experimental details were as described in figure 4.2. Final control cell numbers were $2.62 + 0.5 \times 10^4$/well.
B: Experimental details were as described in figure 4.3. Final control cell numbers were $2.25 + 0.1 \times 10^5$/well.
Values are mean + sd of three determinations.

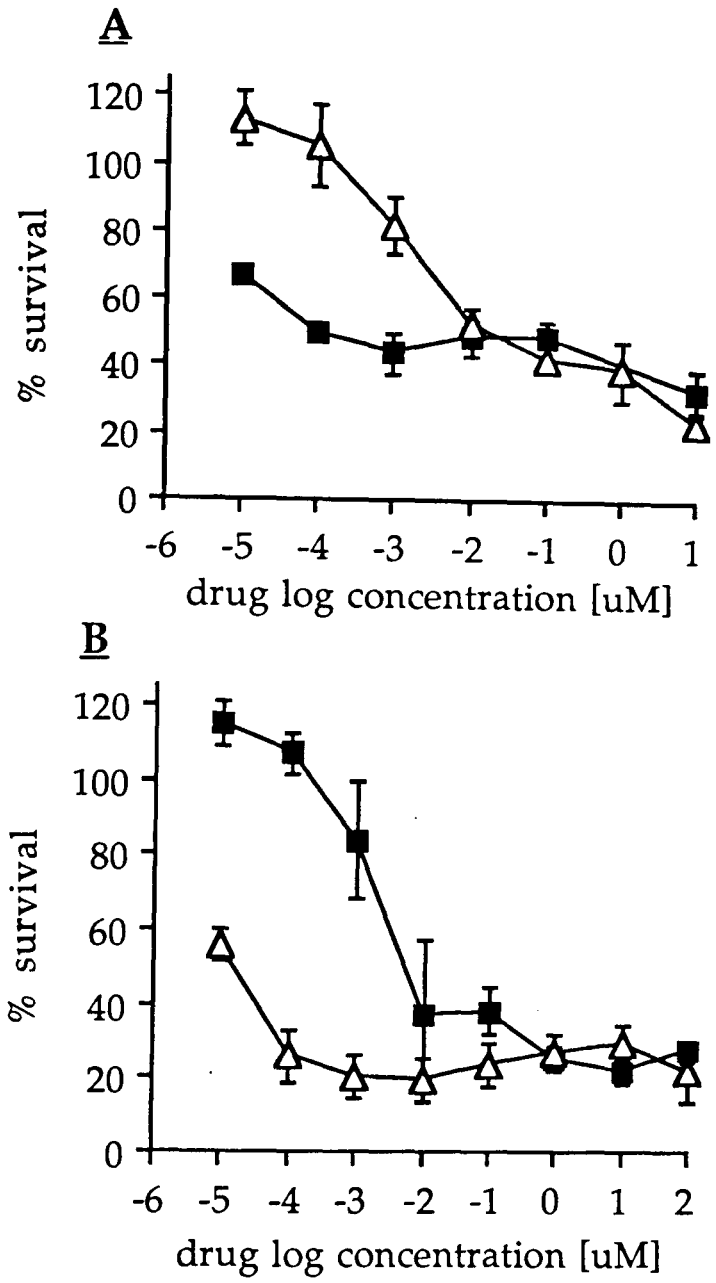


Figure 4.8 *Cytotoxicity of mitozantrone [■] and CI941 [△] in MCF-7 cells after a one hour exposure followed by a further 60 hour drug-free incubation [A] and a further 6 day incubation [B].*

A: Experimental details as figure 4.4. Final control cell numbers were $1.38 + 0.14 \times 10^5$/well. **B:** 10^4 cells were seeded per well and cultured for 24 hours prior to drug exposure. Final control cell numbers were $3.2 + 0.3 \times 10^4$/well. Values are mean + sd of three determinations.

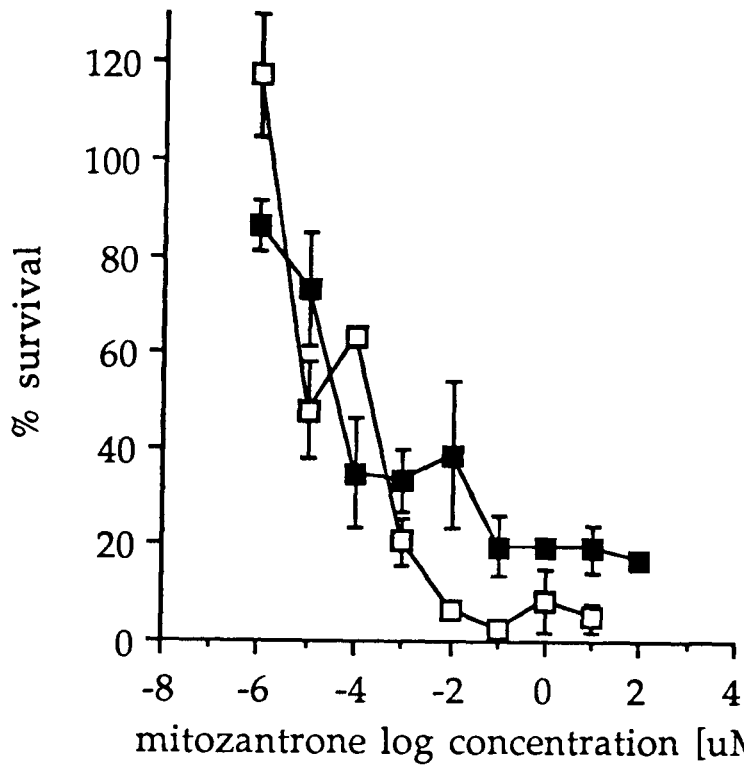


Figure 4.9 *Cytotoxicity of mitozantrone in MCF-7 cells after a one hour exposure followed by a further 11 or 17 days incubation*

[■]- 10^4 cells were seeded per well and cultured for 24 hours prior to drug exposure. Cells were incubated for 11 days after drug exposure. The medium was changed on the fifth day of this incubation. Final control cell numbers were $3.19 + 0.3 \times 10^4$/well.

[□]- Procedure as above. Cells were incubated for 17 days after drug exposure. The medium was changed every five days after drug exposure. Final control cell numbers were $6.27 + 1.4 \times 10^4$/well.
Values are mean + sd of three determinations.

Table 4.2 *Effect of post-exposure incubation time on the LD50 concentration of mitozantrone in MCF-7 cells.*

post exposure incubation time	LD50 concentration [M]
60 hrs	1.3×10^{-7}
144 hrs	5.2×10^{-9}
11 days	4.0×10^{-11}
17 days	1.2×10^{-11}

Exposure time was one hour. Cells/incubate were 10^5 [60 hrs], 10^4 [144 hrs], 10^4 [11days], and 10^4 [17 days]. Data derived from figures 4.8 and 4.9.

Figure 4.10 shows the cytotoxicity of the AQ's in MCF-7 following a 24 hour exposure and immediate determination of the surviving cells. The LD50 values derived from this figure were 1AQ [$13.6x10^{-6}M$], 1,4AQ [$12.4x10^{-6}M$], 1,5AQ [$10.0x10^{-6}M$] ansd 1,8AQ [$6.5x10^{-6}M$]. The effect of further incubating for 96 hours cells exposed to AQ's for 24 hours is shown in figure 4.11. The LD50s derived from this figure were 1AQ [$8.5x10^{-9}M$], 1,4AQ [$5.3x10^{-6}M$], 1,5AQ [$2.6x10^{-6}M$] and 1,8AQ [$1.3x10^{-6}M$].

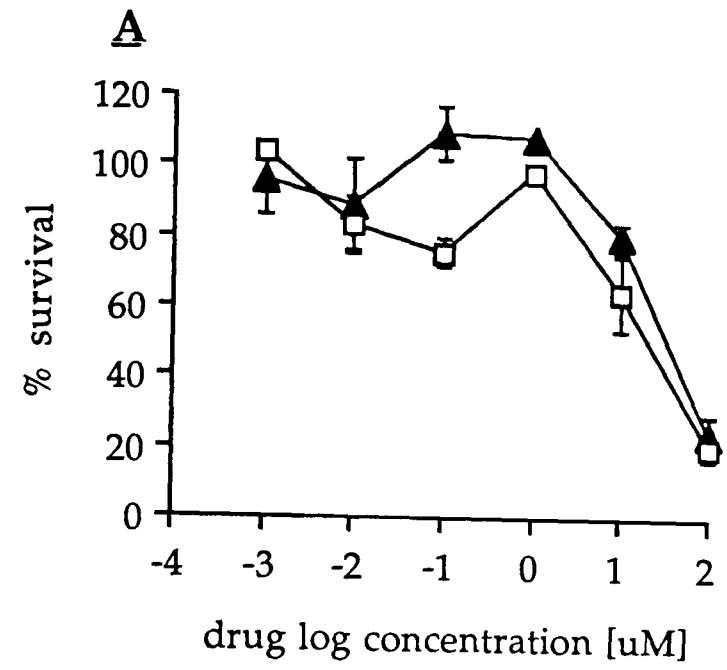
The exposure time was reduced to one hour and the cytotoxicity determined after 6 days further drug free incubation. Figure 4.12 shows the cytotoxicity profile obtained from this study . The LD50 concentrations derived from this data were 1,4AQ [$1.2x10^{-6}M$], 1,5AQ [$12.3x10^{-6}M$] and 1,8AQ [$0.5x10^{-6}M$] (1AQ not determined).

Figure 4.13 shows the cytotoxicity of 1AQ after a one hour exposure followed by 60 or 11 days post-exposure drug-free growth. The LD50 concentrations derived from this figure are shown in table 4.3.

Table 4.4 summarises the LD50 values obtained for the AQ's with the different protocols used.

4.3.1.5 *The effect of oxygen scavengers on the survival of MCF-7 cells exposed to 1AQ, 1,5AQ and 1,8AQ.*

Studies were carried out as described in section 4.2.1. The results obtained are shown in figure 4.14. The AQ concentrations [LD50 values] used were derived from figure 4.12 [1,5AQ and 1,8AQ] and figure 4.13 [1AQ]. 1,4AQ was not used as this drug was found not to redox cycle in MCF-7 cells [see section 2.4.3]. Catalase [6000 units ml^{-1}] restored cell survival to 100% for 1AQ (4uM) and 1,8AQ (0.5uM) which alone produced a survival rate of 37.8 + 4.6% and 39.8 + 3.0% respectively. However, no significant increase in survival was seen with the 1,5AQ. SOD [600ug ml^{-1}] restored cell survival from 39.8 + 3.0 to 83.3 + 2.9% for 1,8AQ, from 28.2 + 3.8 to 36.7 + 1.2% for 1,5AQ and had no effect on



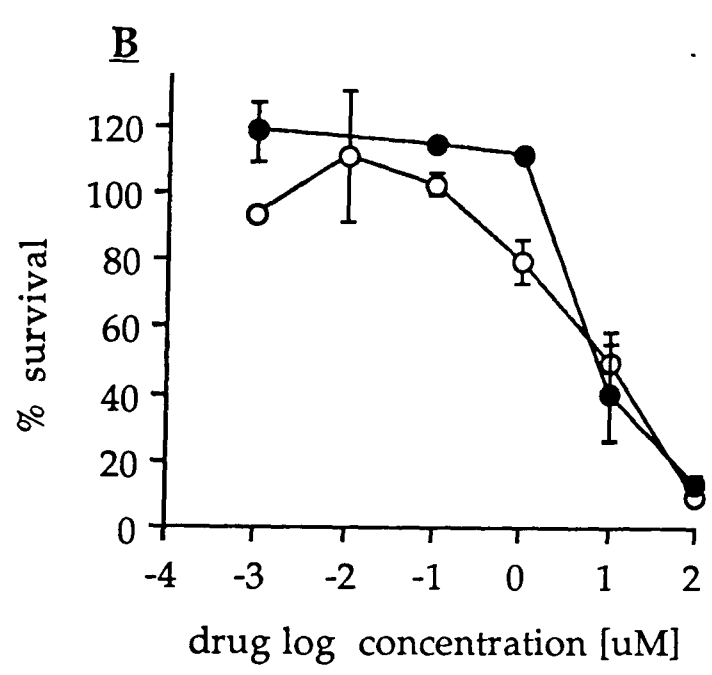


Figure 4.10 *Cytotoxicity of alkylaminoanthraquinones in MCF-7 cells after a 24 hour exposure*

A: [▲] 1AQ and [□] 1,4AQ: B: [○] 1,5AQ and [●]1,8AQ
5×10^4 cells were seeded per well and cultured for 24 hours prior to drug exposure. Final control cell numbers were $3.81 + 0.31 \times 10^4$. Values are mean + sd of three determinations.

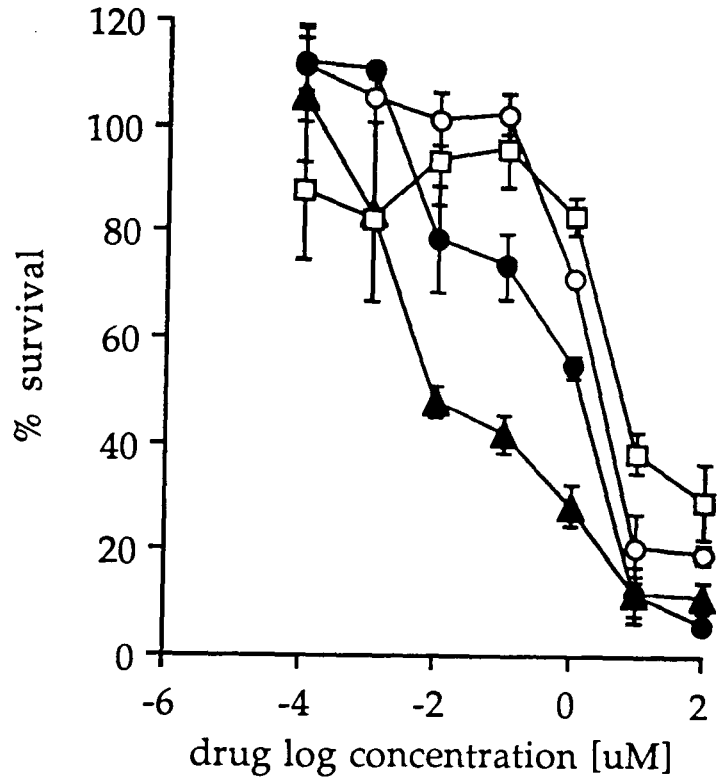


Figure 4.11 *Cytotoxicity of alkylaminoanthraquinones in MCF-7 cells after a 24 hour exposure followed by a further 96 hour incubation.*

Curves are: [▲] 1AQ, [□] 1,4AQ, [○] 1,5AQ and [●] 1,8AQ.
Experimental details were as described in figure 4.3.
Values are mean + sd of three determinations.

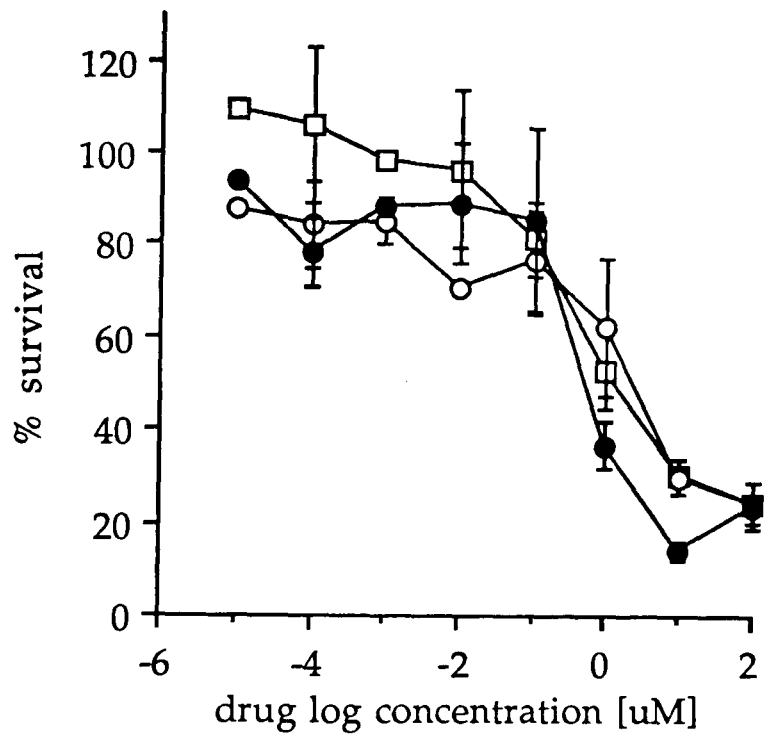


Figure 4.12 *Cytotoxicity of alkylaminoanthraquinones in MCF-7 cells after a one hour exposure followed by a further 6 day drug-free incubation.*

Curves are: [□] 1,4AQ, [○] 1,5AQ and [●] 1,8AQ.
Experimental details were as described in figure 4.8.
Values are mean + sd of three determinations.

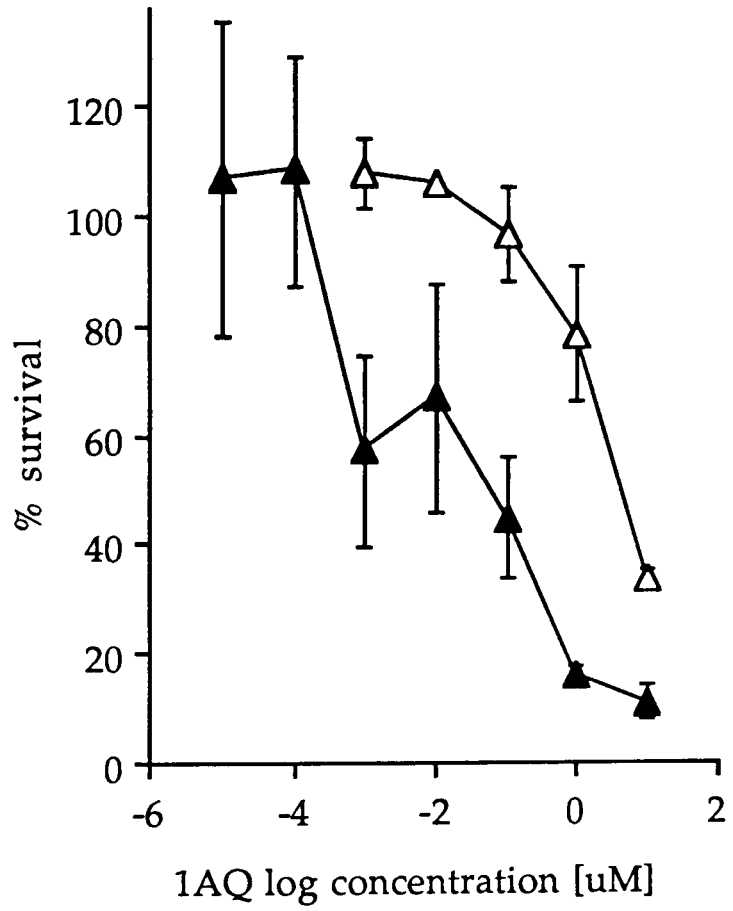


Figure 4.13 *Cytotoxicity of 1AQ in MCF-7 cells after a one hour exposure followed by a further 60 hour [△] or 17 day [▲] incubation*

Experimental details as figure 4.4 and figure 4.9. Values are mean + sd of three determinations.

Table 4.3 *Effect of post-exposure incubation time on the LD50 concentration of 1AQ in MCF-7 cells.*

post exposure incubation time	LD50 concentration [uM]
60 hrs	4.4
11 days	0.06

Exposure time was one hour. Cells/incubate were 10^5 [60 hrs] and 10^4 [11days].

Data derived from figure 4.13.

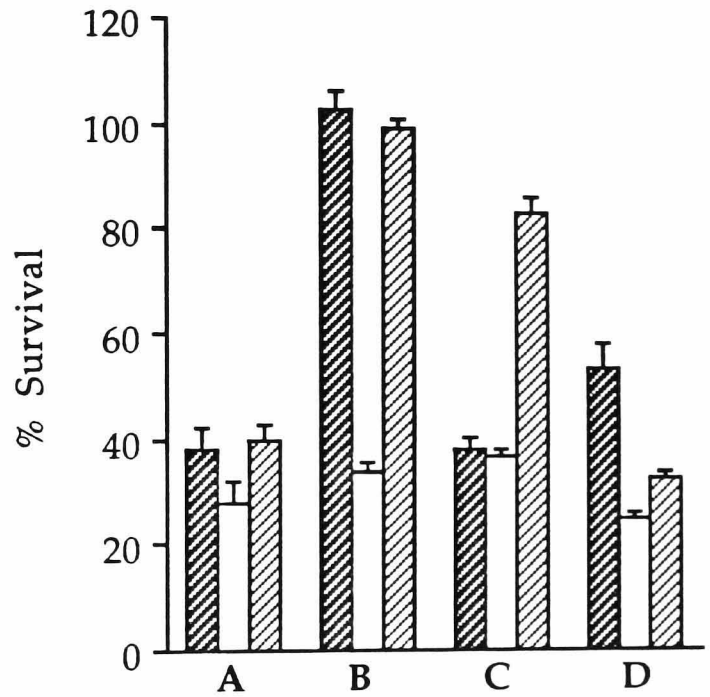


Figure 4.14 *Effect of oxygen radical scavengers on cytotoxicity of 1AQ [▨], 1,5AQ [□] and 1,8AQ [▨] in MCF-7 cells.*

A: Cells were incubated for one hour with 1AQ (4uM), 1,5AQ (12.3uM) and 1,8AQ (0.5uM) or AQ's plus **B**:catalase [6000 units], **C**: SOD [600ug] and **D**: DMSO [200mM].
Experimental details as figure 4.6.
Values are mean + sd of three determinations.

survival of cells exposed to 1AQ. DMSO [200mM] increased survival of cells exposed to 1AQ from 37.8 + 4.6% to 53.2 + 5.0%, but produced no significant increase in survival for 1,5AQ and 1,8AQ.

4.3.2 *Uptake of quinone antitumour agents by MCF-7 cells*

Drug uptake was determined over a ninety minute period as described in section 4.2.2. Figure 3.11 shows that there was no significant decrease in cell survival over this period.

4.3.2.1 *Uptake of doxorubicin, mitozantrone and CI941 by MCF-7 cells*

Figure 4.15 shows uptake of doxorubicin (15uM) by MCF-7 cells [$10^6 ml^{-1}$] in serum supplemented growth medium. There was a rapid association of doxorubicin with cells [5.81 nmole/ 10^6 cells] within the first minute of incubation. Following this time a further 0.87 nmole/10^6 cells was taken up during the ninety minute incubation period.

Figure 4.16 shows the uptake of mitozantrone and CI941 (15uM) by MCF-7 cells. It can be seen that there was a rapid association of drug with the cells within the first minute of incubation with mitozantrone (5.07 + 1.23 nmole) or CI941 (3.96 + 0.56 nmole) [per 10^6cells]. Following this cellular uptake was similar for mitozantrone and CI941 . The amount of drug taken up by the cells after 90 minutes was 6.17 + 0.5 nmole for mitozantrone and 7.16 + 0.63 nmole for CI941 [per 10^6cells].

Mitozantrone uptake by MCF-7 cells was also determined using a polarographic method [section 4.2.2.2]. Figure 4.17 compares the uptake profile obtained for HPLC and polarographic methods. The two systems differed in that uptake was determined in serum supplemented growth medium [15uM drug] prior to HPLC analysis and in phosphate buffered

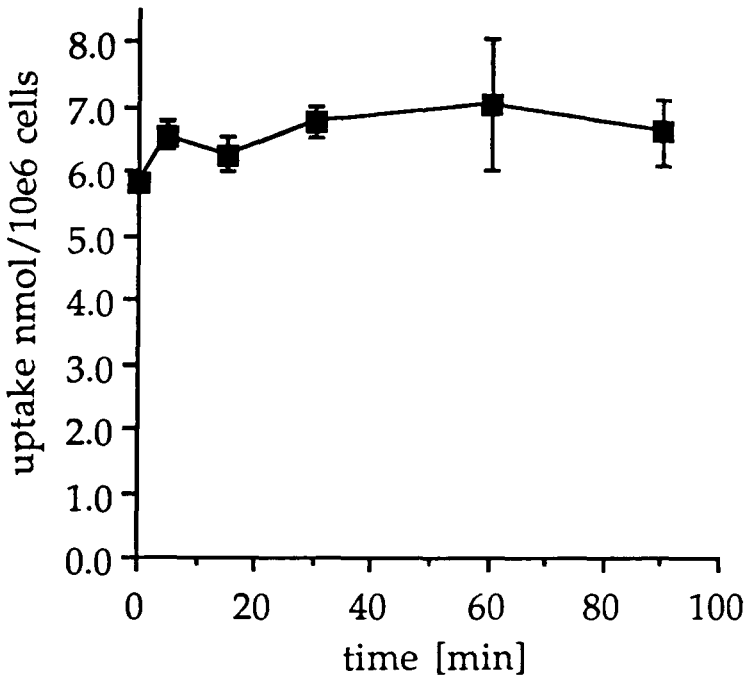


Figure 4.15 *Uptake of doxorubicin by MCF-7 cells.*

Doxorubicin [15uM] was incubated with 10^6 cells ml^{-1}.
Values are mean + sd of three determinations.

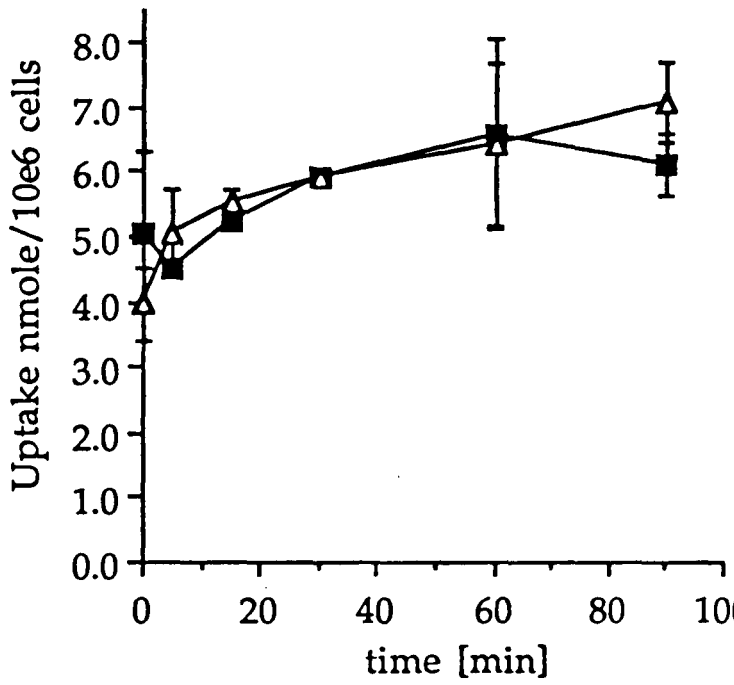


Figure 4.16 *Uptake of mitozantrone [■] and CI941 [△] by MCF-7 cells.*

Drugs [15uM] were incubated with 10^6cells ml^{-1}.
Values are mean + sd of three determinations.

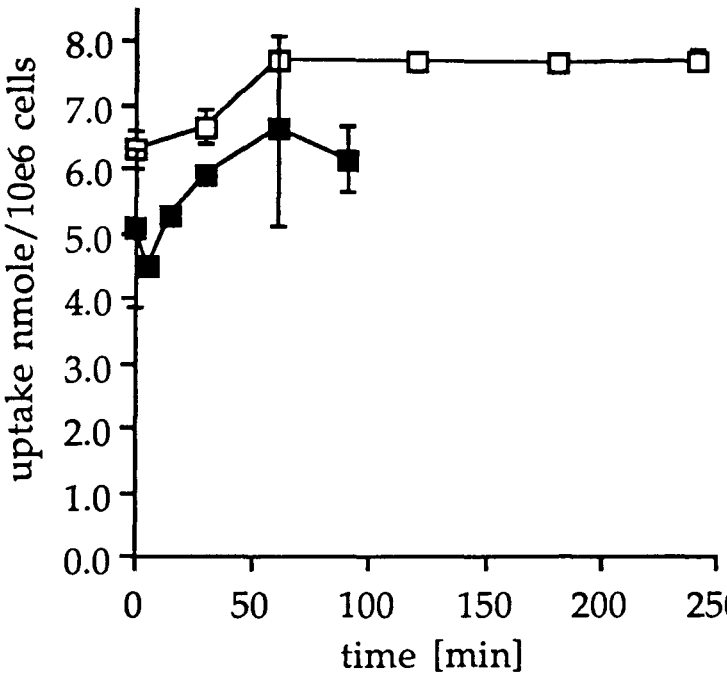


Figure 4.17 *Uptake of mitozantrone by MCF-7 cells as determined by HPLC [□] and polarography [■].*

Drug concentration was 10uM (in PBS) for polarography and 15uM (in RPMI-1640, serum supplemented) for HPLC. 10^6 cells ml^{-1} were used for both protocols. Values are mean + sd of three determinations.

saline [10uM drug] prior to polarographic analysis. The polarographic method showed a slightly higher level of uptake than that determined by HPLC, this may reflect the different conditions used [especially the presence of serum]. The polarographic method however, confirmed that no further uptake was occurring after 60 minutes.

Figure 4.18 shows the viability of the MCF-7 cells [determined by trypan blue exclusion] over 330 minutes in the presence or absence of mitozantrone. This shows that there was no difference between control and drug treated cell survival over this time period. Cell viabilty was still 70% after 330 minutes compared to 90% immediately after harvesting. This indicated that during uptake experiments, cell death and resulting increase in cell permeability was not likely to have contributed to the uptake observed.

4.3.2.2 *Uptake of alkylaminoanthraquinones by MCF-7 cells*

Figure 4.19 shows the uptake profiles of all four AQ's [15uM] by MCF-7 cells. Rapid association of drug with cells was observed within the first minute of incubation for each AQ (nmole/ 10^6cells)- 1AQ (5.51 + 0.89), 1,4AQ (2.88 + 0.2), 1,5AQ (3.09)and 1,8AQ (3.29 + 0.96). Table 4.4 shows the uptake of the AQ's by MCF-7 cells after a one hour exposure. Uptake by 1,8AQ was significantly higher than the other anthraquinones after one hour. However, there was no significant difference in uptake between 1AQ, 1,4AQ and 1,5AQ after one hour.

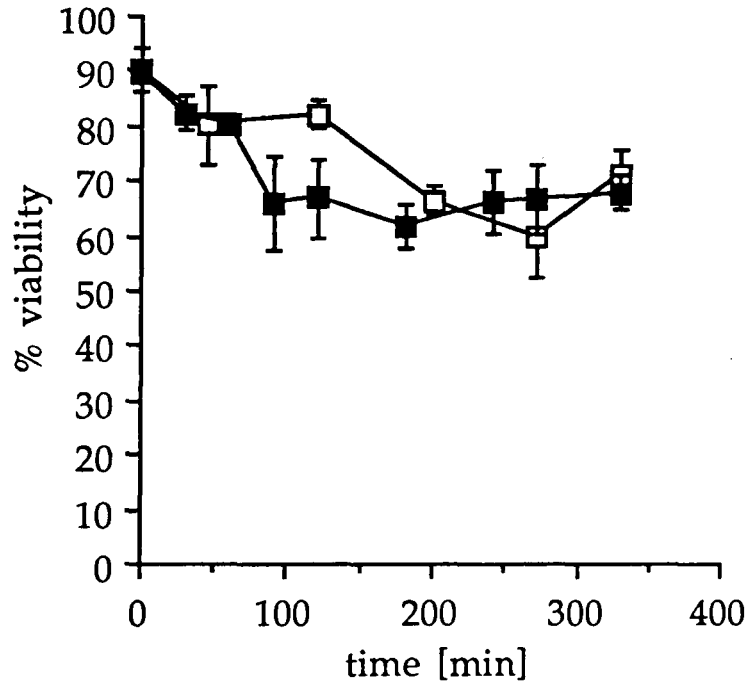


Figure 4.18 *Survival of MCF-7 cells in the presence [■] or absence [□] of mitozantrone (10uM).*

Cell concentration (in PBS) was $10^6 ml^{-1}$. Values are mean + sd of three determinations. Survival was assessed using the trypan blue exclusion assay (appendix A1.9).

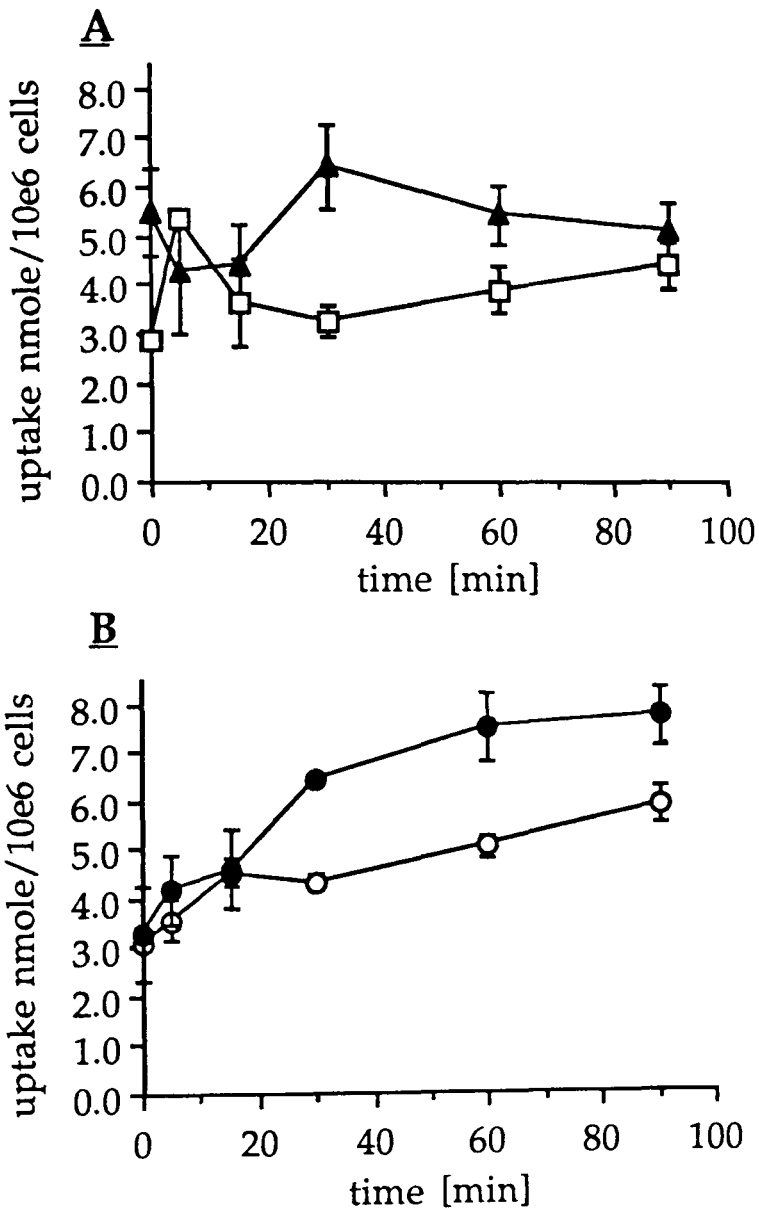


Figure 4.19 *Uptake of alkylaminoanthraquinones by MCF-7 cells*

A: 1AQ [▲], 1,4AQ [□], **B**: 1,5AQ [○] and 1,8AQ [●]. Experimental details as figure 4.15. Values represent mean + sd of three determinations.

Table 4.4 *Uptake of anthraquinones by MCF-7 cells.*

alkylamino-anthraquinone	Uptake nmole/ 10^6cells after 60 minutes
1AQ	5.48 + 0.62
1,4AQ	3.92 + 0.5
1,5AQ	5.1
1,8AQ	7.53 + 0.7

Drug concentration was 15uM. Values are mean + sd of three determinations [except 1,5AQ (2)]

4.4 Discussion

4.4.1 *Cytotoxicity of quinone antitumour agents in MCF-7 human breast cancer cells*

The cytotoxicity of the quinone antitumour agents was determined in MCF-7 cells for several reasons:-

1. To determine whether the MCF-7 cell line was a suitable model system for investigating the mechanism of action of these agents.
2. To enable the cytotoxic potency of quinone based agents to be related to their propensity to redox cycle and produce DNA strand breakage in MCF-7 cells.

As described in section 4.2.1 several different protocols were used to assess cytotoxicity. This enabled a comparison of short and long term cytotoxic effects. Furthermore, this enabled the relationship between exposure time and cytotoxicity to be determined.

LD50 [also known as LC50, EC50, ED50] concentration is the concentration of drug which is required to kill 50% of a cell population. This value is useful when the cytotoxic potency of several drugs need to be compared. Table 4.5 summarises the LD50 concentrations observed when the cytotoxicity of doxorubicin in MCF-7 cells was evaluated using several different protocols. Doxorubicin was least cytotoxic when quiescent cells [fully confluent cells] were exposed for 24 hours [figure 4.2]. This is consistent with doxorubicin cytotoxicity being specific for proliferating cells. The effect of a one hour exposure to doxorubicin was determined because doxorubicin uptake by MCF-7 cells was shown to be maximal after 60 minutes [figure 4.15]. Furthermore, this exposure time may be preferable due to the finding that doxorubicin has a half life of 14 hours in RPMI cell culture medium due to hydrolytic degradation to inactive products such as doxorubicinone (Beijnen *et al.*, 1986). Table 4.5 shows that the LD50 concentration of doxorubicin was

Table 4.5 *Comparison of the cytotoxicity of doxorubicin, mitozantrone and CI941 in MCF-7 cells.*

Exposure time [hrs] + further drug-free growth period	*LD50 concentration [M]* **dox**	**mitoz**	**CI941**
24	0.28×10^{-6}	2.4×10^{-6}	0.8×10^{-6}
24 + 96hrs	0.2×10^{-6}	1.62×10^{-10}	4.6×10^{-10}
1 + 60hrs	5.6×10^{-6}	1.3×10^{-7}	1.6×10^{-8}
1+ 6days	3.0×10^{-6}	5.2×10^{-9}	1.5×10^{-10}
1 + 11days	0.06×10^{-6}	4.0×10^{-11}	nd

nd = not determined

inversely related to the post-exposure incubation period. It is not possible to directly compare the cytotoxicity potency of doxorubicin obtained in this study with that from other studies with MCF-7 cells [see table 4.6] and other cell lines [table 4.7]. This is due to the widely differing protocols used by other workers to determine cytotoxicity. In addition, MCF-7 cells from different laboratories have been shown to have a wide range of biological properties despite possessing the same karyotype, including a ten fold range of tumourogenicity, differences in the level of expression of oestrogen and progesterone receptors, differences in growth rate and differences in response to hormones (Osborne *et al.*, 1987). These parameters could infuence the response of these cells to cytotoxic agents. In the present study, cell kill by doxorubicin was also dependent on the cell growth period after drug exposure. This is due to a larger difference between the number of control cells and treated cells becoming evident reflecting an inhibition of the proliferative ability of the treated cells. The results of this study would suggest that use of a short or no post-exposure incubation period gives an underestimate of cytotoxic potential.

The cytotoxicity of mitozantrone and CI941 in MCF-7 cells was determined in order to compare their cytotoxic potency with that of doxorubicin. Table 4.5 compares the LD50 concentrations obtained for all three compounds. The LD50 of mitozantrone and CI941 was inversely related to the post-exposure incubation period. This suggests that there is a time delay before mitozantrone and CI941 cell kill is expressed. The potency of these two agents was consistently greater than doxorubicin in a series of one hour exposures with increasing post-exposure incubation periods [see table 4.5]. These results are consistent with the finding that mitozantrone is more cytotoxic than doxorubicin in other tumour cell lines [Dalton *et al.*, 1988; Epstein *et al.*, 1988; Drewinko *et al.*, 1983; Cowan *et al.*, 1983; Mergenthaler *et al.*, 1987]. Graham *et al.* (1989) found that the LD50 of CI941 in CCRF-CEM cells was 8.0×10^{-9}M, a result consistent with the potency of CI941 in MCF-7 cells. The LD50 obtained for studies on mitozantrone cytotoxicity in a variety of human tumour cells are shown in table 4.8. However, no studies have previously been reported in MCF-7 cells. The data in

Table 4.6 *Cytotoxicity of doxorubicin in MCF-7 human breast cancer cells as determined in other studies*

Exposure time + further drug-free growth period	ED50 [uM]	Reference
24hrs + 10-12 days	5.0	Dusre *et al* (1989)
1hr + 12 days	0.5	Doroshow (1986)
3days	0.005	Mimnaugh *et al* (1989)
7days	0.025	Batist *et al* (1986)
3 days	0.017	Yusa *et al* (1988)

Table 4.7 *Cytotoxicity of doxorubicin in human tumour cell lines*

Cell line	Exposure time + further drug-free growth period	ED50 [M]	Reference
Human colon carcinoma	1hr + 10days	5.4×10^{-8}	Dalton *et al.*, (1988)
T-47D human human breast carcinoma	1day + 5days	9.25×10^{-6}	Epstein *et al.*, (1988)
Human colon carcinoma	1hr + 21days	3.7×10^{-7}	Drewinko *et al., (1983)*
Human lung adenocarcinoma A549	1hr + 6days	1.9×10^{-7}	Lawrence (1988)
Human myeloma RPMI-8226	1hr + 10-14days	10^{-8}	Bellamy *et al.*, (1988)
Ehrlich ascites carcinoma	1hr + 6 days	1.5×10^{-6}	Doroshow (1986)
Human lung tumour SW1573	1hr + 7-10days	0.5×10^{-6}	Keizer *et al.*, (1988)
Ovarian carcinoma A2780	2hr + 3days	0.3×10^{-6}	Cervantes *et al.*, (1988)
Hattori human breast cancer	3 days	17.0×10^{-9}	Yusa (1988)

table 4.8 demonstrates that mitozantrone is an effective antitumour agent *in vitro*, although for reasons previously stated it is not reasonable to directly compare the cytotoxic potency of mitozantrone observed in different cell lines. Table 4.5 shows that CI941 was consistently more cytotoxic than mitozantrone following a one hour exposure.

The cytotoxicity of the alkylaminoanthraquinones in MCF-7 cells was determined in a similar manner to that for doxorubicin, mitozantrone and CI941. The results are summarised in table 4.9. Partridge (1985) studied the cytotoxicity of these agents in human leukaemic cells [HeLa] following a 24 hour exposure and found the order of cytotoxic potency to be (LD50uM):- 1,4AQ(1.0) > 1,5AQ(3.0) > 1,8AQ(6.0) > 1AQ(20.0). This order of potency is different to that found in the present study (see table 9). This is possibly due to differences between the levels of enzymes critical for AQ action in these cell lines, for example topoisomerases [see section 1.7.1] and/ or reductase enzymes [see section 1.8.1]. The AQ's are clearly less potent than mitozantrone and CI941 in the MCF-7 cell line [compare table 4.9 and table 4.5]. Drewinko *et al.* (1983) and Nishio and Uyeki (1983) also found that 1,4AQ was less cytotoxic than mitozantrone. In the present study, 1,4AQ, 1,5AQ and 1,8AQ were found to be less cytotoxic than doxorubicin in the MCF-7 cell line. However, 1AQ was the most cytotoxic AQ in the present study, being more cytotoxic than doxorubicin in several of the protocols used (compare table 4.5 and 4.9).

The different cytotoxic potentials of these quinone antitumour agents in the MCF-7 cell line clearly points to them having different modes of action or different propensities to exert a similar mode of action. This is discussed in chapter 5 with relation to the ability of these agents to redox cycle, inhibit MCF-7 topoisomerase I and produce DNA strand breakage in the MCF-7 cell line.

4.4.2 *Uptake of quinone antitumour agents by MCF-7 cells*

Table 4.8 *Cytotoxicity of mitozantrone in human tumour cell lines*

Cell line	Exposure time + further drug-free growth period	ED50 [M]	Reference
Human colon carcinoma	1hr + 10days	3.2×10^{-8}	Dalton *et al.*, (1988)
T-47D human breast carcinoma	1day + 5days	1.1×10^{-6}	Epstein *et al.*, (1988)
Human haematopoietic cell lines	2days	2.3×10^{-9}	Roos (1987)
Malignant mesenchymal tumour cells	3days	2.0×10^{-8}	Dietel *et al.*, (1988)
Human cancer (HeLa)	24hrs	1×10^{-6}	Partridge (1987)

Table 4.9 *Comparison of the cytotoxicity of alkylaminoanthraquinones in MCF-7 cells.*

Exposure time [hrs] + further drug-free growth period	LD50 concentration [M]			
	1AQ	1,4AQ	1,5AQ	1,8AQ
24	13.6×10^{-6}	12.4×10^{-6}	10.0×10^{-6}	6.5×10^{-6}
24 + 96hrs	8.5×10^{-10}	5.3×10^{-6}	2.6×10^{-6}	1.3×10^{-6}
1 + 60hrs	4.4×10^{-6}	nd	nd	nd
1+ 6days	nd	1.2×10^{-6}	12.3×10^{-6}	0.5×10^{-6}
1 + 11days	6.0×10^{-8}	nd	nd	nd

nd = not determined

The cytotoxicity of quinone antitumour agents in MCF-7 cells has been discussed. To investigate whether or not any differences in the cytotoxicity observed could be accounted for by differences in the amounts of drugs taken up by MCF-7 cells, a series of drug uptake studies were carried out. Table 4.10 shows that there was no significant difference in the amount of doxorubicin, CI941 and mitozantrone taken up by the MCF-7 cells. Uptake of all three of these compounds reached steady state after one hour [see section 4.3.2.1]. Moreover, drug uptake into MCF-7 cells was not related to cell death and concomitant loss of membrane integrity of MCF-7 cells during the uptake assay [see figure 3.11]. Similar to the present study other researchers found doxorubicin uptake to reach steady state between 15 and 60 minutes [Skovsgaard, 1978; Friche *et al.*, 1987; Paneerselvam, 1987; Broggini *et al.*, 1988; Kessel and Wilberding, 1985] and mitozantrone uptake reached steady state after 30 minutes (Burns *et al.*, 1987; Nishio and Uyeki, 1983). Doxorubicin has been shown to diffuse into cells by passive diffusion in the unionised form [reviewed by Siegfried *et al.*, 1985]. However, it has also been suggested that the uptake of doxorubicin is by facilitated diffusion due to the high affinity of doxorubicin for intracellular binding sites driving the diffusion gradient (reviewed by Siegfried *et al.*, 1985). This uptake is opposed by an energy requiring efflux mechanism in some cell lines (Skovsgaard, 1977 and 1978; Inaba and Johnson, 1978; Dano, 1973), a process that has been found to be enhanced in resistant cells [reviewed by Siegfried *et al.*, 1985]. A corresponding increase in the expression of a 170,000 dalton plasma membrane protein, putatively involved in active efflux has also been found in resistant cells [reviewed by Siegfried *et al.*, 1985; Beck, 1987]. It is not known whether such an efflux system is present in MCF-7 cells or whether if such a system exists it is capable of removing other quinone antitumour agents such as mitozantrone, CI941 and AQ's.

In this study, similar drug uptake profiles were observed for the alkylaminoanthraquinones, doxorubicin, CI941 and mitozantrone [see table 4.10]. The uptake of the AQ's also reached steady state after one hour, the slight differences in cell uptake observed not being sufficient to explain the differences in cytotoxicity of these agents.

Table 4.10 *Comparison of uptake of quinone antitumour drugs by MCF-7 cells.*

Compound	Uptake nmole/ 10^6cells 0 minutes	60 minutes
doxorubicin	5.81*	7.08 + 1.02
mitozantrone	5.07 + 1.23	6.63 + 1.5
CI941	3.96 + 0.56	6.47 + 1.26
1AQ	5.51 + 0.89	5.48 + 0.62
1,4AQ	2.88 + 0.2	3.92 + 0.5
1,5AQ	3.09*	5.1*
1,8AQ	3.29 + 0.96	7.53 + 0.7

Drug concentration was 15uM. Values are mean + sd of at least three determinations [except *(2)].

A phenomenon observed for all the quinone antitumour agents was a rapid association of drug with the cells within the first seconds of the incubation. This effect was observable visually, as the cell pellet was distinctly coloured by the drugs. This effect is likely to be due to a rapid binding of drug to the cell membrane. Doxorubicin [reviewed by Gianni *et al.*, 1983] and mitozantrone [Taylor *et al.*, 1981] have been shown to bind to membrane components with high affinity [see section 1.16 and 1.17]. After this rapid association , only a small amount of further drug uptake was observed [see figures 4.15,4.16 and 4.19]. This binding effect has also been shown for other cell lines with both doxorubicin [Inaba and Johnson 1978; Skovsgaard 1978; Paneerselvam 1987] and mitozantrone [Nishio and Uyeki, 1983; White and Durr, 1985; Wallace, 1987]. However, many workers have assumed that drug uptake is zero at time 0 and consequently have missed this component of drug interaction with the cell. Rapid association of drug with the cell membrane is probably followed by diffusion of drug into the cell due to a gradient facilitated by the affinity of quinone antitumour agents for cellular components such as DNA. Indeed, despite this apparent extracellular membrane binding, doxorubicin is found to rapidly relocate mainly in the nucleus of the cell (reviewed by Gianni *et al.*, 1983).

4.4.3 *The effect of oxygen free radical scavengers on the cytotoxicity of doxorubicin, 1AQ, 1,5AQ and 1,8AQ.*

The results in chapter 2 showed that doxorubicin, 1AQ, 1,5AQ and 1,8AQ are capable of redox cycling in MCF-7 cells. Furthermore, these agents all formed highly reactive, cell damaging hydroxyl radicals in NADPH-fortified MCF-7 S9 fraction as determined by spin trapping techniques [see figure 2.23]. In addition, hydroxyl radicals were detected in an incubate of doxorubicin with intact viable MCF-7 cells [see figure 2.9]. Mitozantrone, CI941 and 1,4AQ were not included in these studies as these compounds did not redox cycle or produce hydroxyl radicals in the above S9 system [see section 2.4.2 and 2.4.3]. To determine whether the formation of hydroxyl radicals by these redox cycling agents was related to their cytotoxic action in MCF-7 cells, the effect of oxygen free radical scavengers on the cytotoxicity of these agents was

investigated (as described in section 4.2.1). DMSO and catalase produced a slight increase in the survival of cells treated with an LD50 concentration of doxorubicin [3.0uM] (figure 4.5). This is probably due to DMSO scavenging hydroxyl radicals and catalase preventing the formation of hydroxyl radicals by scavenging hydrogen peroxide. This would inhibit hydroxyl radical formation by preventing hydrogen peroxide from entering the iron catalysed Haber-Weiss reaction [equations 1,2 and 3]. The oxygen radical scavengers N-acetyl cysteine and superoxide dismutase did not influence MCF-7 survival after treatment with doxorubicin. When the concentration of oxygen free radical scavengers were increased, catalase, superoxide dismutase and catalase plus superoxide dismutase increased MCF-7 cell survival to a small extent [figure 4.6]. However, a higher concentration of DMSO had no significant effect on cell survival. SOD scavenging activity can be rationalised by its involvement in inhibiting superoxide anion reduction of iron(III) required for the iron catalysed Haber-Weiss reaction [equation 1].

$$Fe^{3+} + O_2^{-} \rightleftharpoons Fe^{2+} + O_2 \qquad (1)$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + OH\cdot \qquad (2)$$

$$\text{net:}\quad O_{2-}\cdot + H_2O_2 \longrightarrow O_2 + OH\cdot + OH^- \qquad (3)$$

The inability of the oxygen free radical scavengers to protect MCF-7 cells totally against doxorubicin cytotoxicity suggests that doxorubicin mediated oxygen radical generation is occurring at a site inaccessible to these scavengers. It is also possible that doxorubicin mediated oxygen free radical formation is not entirely responsible for its cytotoxic action in MCF-7 cells. These results are consistent with the inability of the large molecular weight enzymes SOD and catalase to permeate the cell membrane. This is supported by the work of Cervantes *et al.* (1988), who found that only high concentrations of hydroxyl radical scavengers which were capable of permeating the cell membrane could protect human ovarian cancer cells against doxorubicin cytotoxicity. In this study, DMSO, may not be reaching the site of hydroxyl radical generation at a sufficient concentration to provide sufficient protection whilst at higher concentrations [200mM] this scavenger could be damaging other cellular properties such as membrane permeability. Other workers have shown that SOD, catalase or DMSO produced a more profound inhibition of doxorubicin cytotoxicity in MCF-7 cells (Doroshow, 1986a) and Ehrlich ascites tumour cells (Doroshow, 1986b). The denatured enzymes were found to have no significant effect.

SOD and catalase increased the survival rate of MCF-7 cells treated with 1,8AQ (see figure 4.14). Similarly, catalase increased MCF-7 cell survival in cells treated with 1AQ (figure 4.14). However, as with doxorubicin, DMSO [200mM] failed to protect against the cytotoxicity of 1AQ or 1,8AQ. SOD, catalase and DMSO provided no protection for MCF-7 cells against the cytotoxicity of 1,5AQ. The protection against 1AQ and 1,8AQ cytotoxicity provided by catalase, a macromolecule that presumably cannot penetrate the cell membrane, would suggest that this enzyme is

scavenging hydrogen peroxide (see equations 2 and 3) which is either being produced externally to the cell or is diffusing out of the cell. Similarly, SOD would appear to be scavenging extracellularly produced superoxide anions, since this charged species is too polar to be able to cross the cell membrane. The failure of SOD to protect MCF-7 cells against 1AQ cytotoxcity could be explained by an absence of extracellular superoxide anion formation by this agent. The lack of protection of these oxygen radical scavenging enzymes against 1,5AQ cytotoxicity could indicate that oxygen free radical formation mediated by this drug could be occurring at intracellular sites inaccessible to these enzymes.

These results could have important implications in rationalising the site(s) of enzymic activation of AQ's to produce reactive oxygen species by redox cycling and the site(s) where these reactive species produce cellular damage.

CHAPTER 5

General Discussion

CHAPTER 5

General Discussion

5.1 *Redox cycling by quinone antitumour agents in MCF-7 cells*

This study has investigated the ability of quinone antitumour agents to undergo flavin reductase mediated redox cycling in MCF-7 human breast cancer cells. Doxorubicin, an anthracycline antitumour agent, was used as a reference in these studies since this agent has previously been shown to redox cycle in human (Basra *et al.*, 1985) and animal, liver and heart tissues (reviewed by Gianni *et al.*, 1983). In the present study doxorubicin was found to redox cycle in the MCF-7 cell line with resultant formation of hydroxyl radicals [see section 2.4.1]. Figure 5.1 shows a schematic diagram of flavin reductase mediated doxorubicin redox cycling. Redox cycling by doxorubicin, correlated with the formation of DNA strand breaks [section 3.4.4.1] and the cytotoxicity [4.4.1] of doxorubicin in MCF-7 cells. A role for hydroxyl radicals in doxorubicin mediated MCF-7 cell DNA strand breakage was supported by the production of plasmid DNA strand breakage by doxorubicin in the presence of purified xanthine oxidase and cytochrome P450 reductase [section 3.4.1.1]. Further evidence for doxorubicin free radical mediated cell damage in MCF-7 cells was provided by the finding that the reactive oxygen scavengers superoxide dismutase, catalase and DMSO partially protected against doxorubicin mediated MCF-7 cell kill [see section 4.4.3]. The lack of complete protection was likely to be due to the inaccessibility these scavengers to some intracellular sites of reactive oxygen formation [as discussed in section 4.4.3]. The results obtained for doxorubicin indicate that the MCF-7 cell line is suitable for the investigation of metabolic activation of quinone antitumour agents and its cellular consequences.

Mitozantrone and CI941 were developed with a view to producing antitumour agents with reduced cardiotoxicity compared to doxorubicin [see section 1.14], a deleterious toxic effect attributed to redox

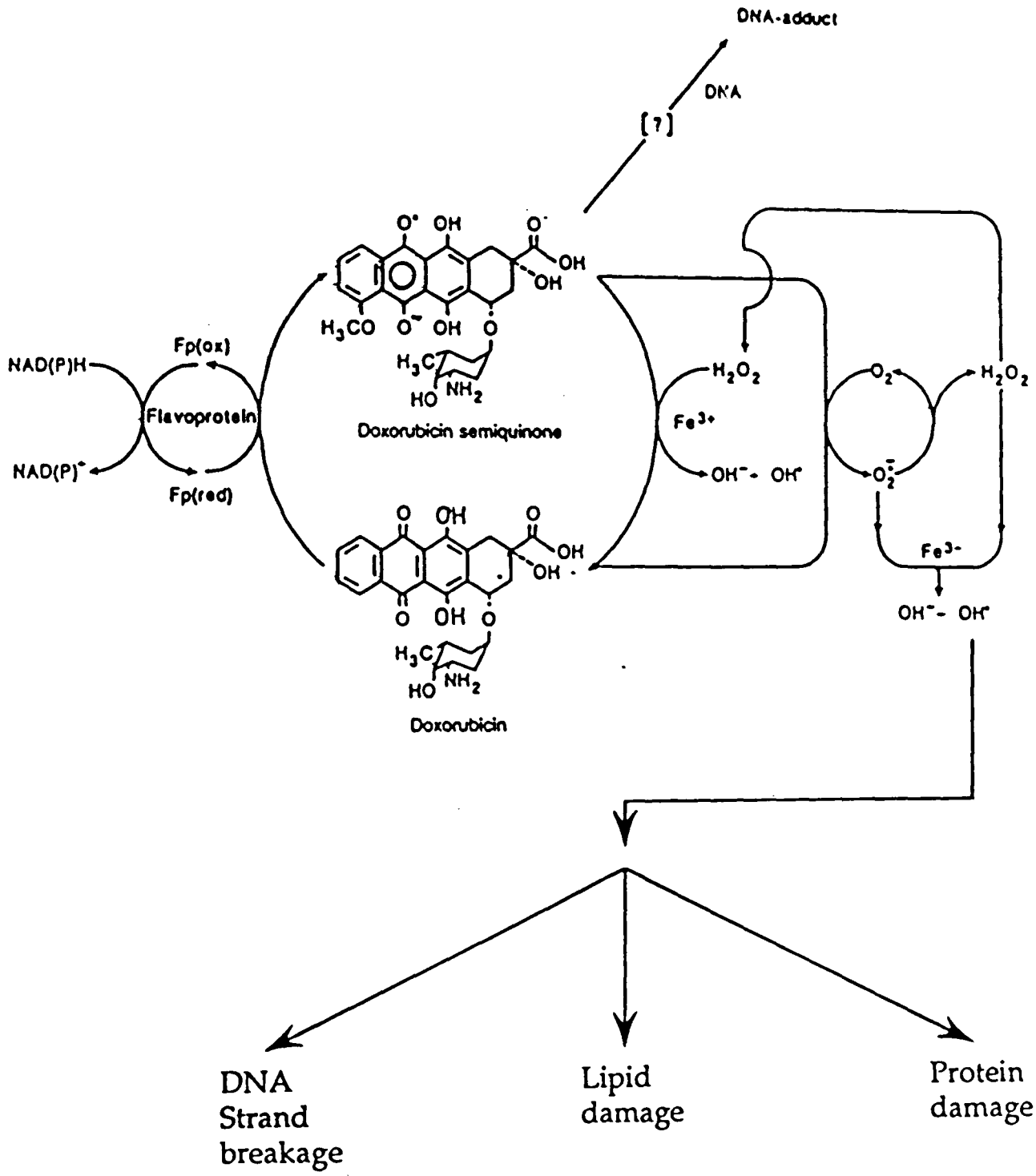


Figure 5.1 *Reductase mediated redox cycling by doxorubicin*

cycling in heart tissue [see section 1.16.5]. In the present study mitozantrone and CI941 were found not to be metabolically activated in MCF-7 cells. In addition, CI941 did not produce plasmid DNA strand breakage in the presence of purified xanthine oxidase and NADPH cytochrome P450 reductase [see section 3.4.1.2]. However, in contrast mitozantrone did produce plasmid DNA strand breakage in the presence of cytochrome P450 reductase [section 3.4.1.2] showing that this compound can be activated by this enzyme when it is removed from its normal cellular membrane environment. Despite the absence of redox cycling in MCF-7 cells, mitozantrone and CI941, at a lower concentration than doxorubicin, both produced an equivalent amount of DNA strand breakage [3.4.4.1]. Furthermore, mitozantrone and CI941 were considerably more cytotoxic than doxorubicin in MCF-7 cells [see table 4.5].

This study also investigated redox cycling in MCF-7 cells by a series of alkylaminoanthraquinones based on mitozantrone (figure 1.35). 1AQ, 1,5AQ and 1,8AQ were found to redox cycle in MCF-7 cells with resultant formation of hydroxyl radicals [section 2.4.3]. These agents were also able to mediate strand breakage of plasmid DNA in the presence of purified NADPH cytochrome P450 reductase [3.4.1.3]. Hydroxyl radical formation in MCF-7 cell S9 fraction and plasmid DNA strand breakage *in situ* correlated with the production of frank DNA strand breakage in MCF-7 cells (at 100uM) [section 3.4.4.2]. It is therefore likely that the cytotoxicity [section 4.4.1] of these compounds in MCF-7 cells is mediated by redox cycling mediated hydroxyl radical formation and resultant DNA strand breakage. As would be expected, decreasing the drug concentration and hence the amount of hydroxyl radicals formed, resulted in less DNA strand breakage in MCF-7 cells [section 3.4.4.2]. A role for redox cycling in the mechanism of action of these agents was further supported by the protective effect of superoxide dismutase and catalase against the cytotoxicity of 1AQ and 1,8AQ in MCF-7 cells [see section 4.4.3]. However, these oxygen radical scavengers did not protect MCF-7 cells against the cytotoxic effect of 1,5AQ, suggesting that the site of free radical formation by this compound was inaccessible to these enzymes [as discussed in section 4.4.3]. Despite being redox active and producing hydroxyl radicals, these compounds (at LD50 concentrations) produced an equal amount of DNA strand breakage to

mitozantrone and CI941. Furthermore, these compounds are considerably less cytotoxic than mitozantrone and CI941 in MCF-7 cells [compare tables 4.5 and 4.9].

In contrast to 1AQ, 1,5AQ and 1,8AQ, the 1,4AQ derivative was found not to redox cycle in MCF-7 cells [2.4.3]. In addition, this compound (100uM) produced less cellular DNA strand breakage in MCF-7 cells [section 3.4.4.2]. Furthermore, this compound did not mediate strand breakage of plasmid DNA in the presence of purified reductases [section 3.4.1.3]. However, despite the absence of redox cycling, this compound (at an LD50 concentration) produced an equal amount of DNA strand breakage to the other alkylaminoanthraquinones and the cytotoxicity of this compound was similar to that of the 1,5AQ and 1,8AQ [see table 4.9]. Differences in cellular uptake could not explain the different levels of strand breakage and cytotoxicity caused by the agents in the present study (see section 4.4.2).

As described in section 2.4 the reductase mediated redox cycling observed for doxorubicin, 1AQ, 1,5AQ and 1,8AQ is likely to result in the formation of hydroxyl radicals via the iron catalysed Haber-Weiss reaction [see section 2.4.1]. However, in order to relate this redox activity to the DNA strand breakage observed *in vitro* for these compounds several points need to be considered:-

(1) The hydroxyl radical is a highly reactive species which can only diffuse 5-10 molecular diameters before it reacts [Pryor, 1988]. Therefore, in order to produce DNA damage there is a requirement for it to be produced in close proximity to the target site. In the case of MCF-7 cell DNA strand breakage the target is presumably the nucleosome. Nuclear activation of quinones has been shown to be feasible in some cell types, due to the presence of cytochrome P450 reductase in the nuclear envelope [Romano, 1983; Bachur, 1979]. The less reactive products of quinone redox cycling, namely superoxide anions and hydrogen peroxide, can diffuse further than hydroxyl radicals and could therefore participate in the iron-catalysed Haber-Weiss reaction mediated

formation of hydroxyl radicals in close proximity to the DNA. DNA strand breakage could therefore occur at sites distant from the original site of reductase mediated activation of the quinone. Furthermore, the products of membrane lipid peroxidation have been shown to mediate DNA strand breakage [Hruszkiewycz, 1988; reviewed by Vaca, 1988]. In support of this doxorubicin has been shown to stimulate lipid peroxidation in animal tissues (reviewed by Powis, 1989).

Spin trapping studies in intact viable MCF-7 cells revealed that doxorubicin-mediated hydroxyl radical formation appeared to be occuring extracellularly (section 2.3.1.6) due to the finding that the DMPO-OH adduct observed persisted upon removal of cells from the incubate. This suggests that the DMPO-OH adduct is diffusing out of the cell or that reactive oxygen species are being formed extracellularly since superoxide anions and hydroxyl radicals are unable to traverse the cell membrane due to their charge and/or reactivity. However, it is also possible that the doxorubicin semiquinone free radical may effectively be transferred across the cell membrane by a process involving electron transfer between doxorubicin molecules bound within the membrane [see figure 5.2]. Alternatively, the doxorubicin semiquinone free radical or hydrogen peroxide may be able to cross the cell membrane (figure 5.2). This is supported by the work of Ramakrishna *et al.* (1988a and 1988b) who showed that the radical anions of 5-nitrofurans, 2 and 5-nitroimidazoles and the benzosemiquinone of 1,4-benzoquinone were formed intracellularly but could only be be detected extracellularly in rat hepatocytes. A further possibility for the presence of extracellular DMPO-OH is that the doxorubicin semiquinone free radical may be produced extracellularly via comproportionation (see figure 5.2 and section 1.8.2). However, this supposes that a sufficient amount of the fully reduced doxorubicin metabolite is formed and can diffuse into the extracellular milieu (see section 1.8.2).

The occurrence of extracellular hydroxyl radical spin adduct (DMPO-OH) by the mechanisms outlined above is further indicative that the free radicals produced intracellularly by flavin reductase mediated doxorubicin redox cycling are able to traverse the nuclear membrane.

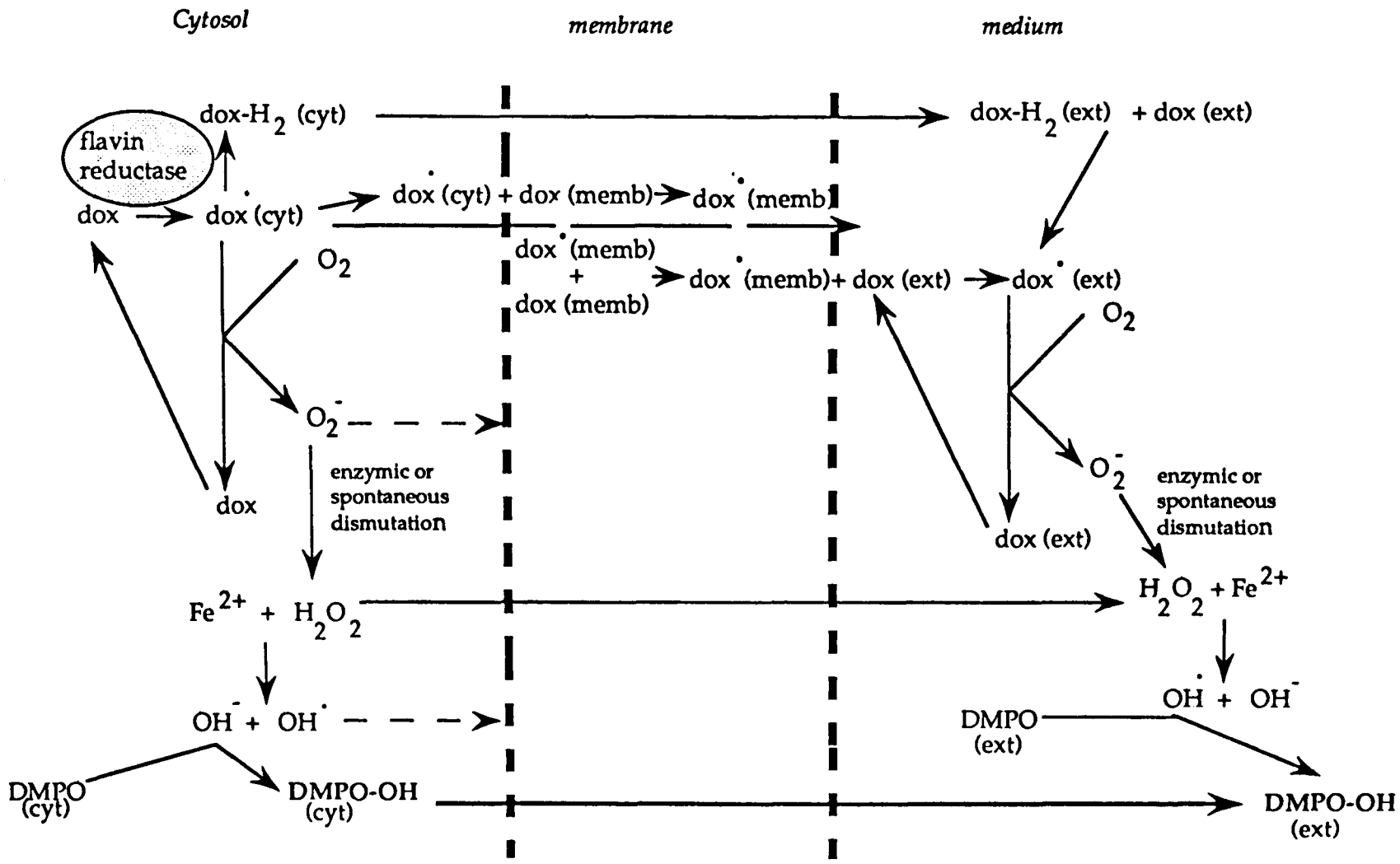


Figure 5.2 *Schematic diagram showing the possible mechanisms of extracellular DMPO-OH adduct formation in the presence of doxorubicin*

dox=doxorubicin
dox-H_2 = fully reduced doxorubicin
dox·=doxorubicin semiquinone
cyt=cytosol
memb=membrane
ext=extracellular

Furthermore, the lack of full protection by oxygen free radical scavengers against doxorubicin mediated MCF-7 cell kill, is further supportive that flavin reductase mediated doxorubicin redox cycling and resultant cell damage is occurring at intracellular sites [see section 4.4.3].

(2) The second consideration is whether iron is available *in vivo* to catalyse the Haber-Weiss reaction as free iron is virtually absent in biological systems. Within the cell iron is stored in the protein ferritin which consists of a protein shell (apoferritin) enclosing iron in the form of a hydrated iron(III)oxide-phosphate complex. The protein shell is made up of 24 subunits and has pores that allow access to the interior (reviewed by Halliwell and Gutteridge, 1984). There is also a small pool of non-protein bound iron which is found attached to chelating agents such as ATP, GTP and citrate which could be available for the Haber-Weiss reaction since these have been shown to react with hydrogen peroxide *in vitro* [Floyd, 1983; Vile *et al.* 1987]. Furthermore, some free iron is likely to be found bound to DNA phosphate groups as these have high affinity for iron. Recent evidence has indicated that doxorubicin can release iron from cellular storage depots including ferritin (Demant, 1984; Thomas and Aust, 1986) and non-ferritin bound microsomal iron pools (Minotti, 1989). This reaction proceeds via reduction of ferritin bound iron(III) to iron(II) mediated by the semiquinone free radical or superoxide anions which enter the pores of apotransferrin. More recent evidence has suggested that quinones with one electron reduction potentials more negative than that of ferritin [-0.24V) can release ferritin bound iron (Monteiro *et al.*, 1989). Therefore, it is possible that the redox cycling alkylaminoanthraquinones of the present study would be able to release ferritin bound iron (see table 2.3 for reduction potentials). Thus it seems likely that iron is available *in vivo* to catalyse hydroxyl radical formation via the Haber-Weiss reaction during quinone antitumour agent redox cycling [see section 1.8.2]. Furthermore, some tumour types are rich in deposits of iron, including some human breast tumours and tumour nodules in Hodgkins disease (reviewed by Halliwell and Gutteridge, 1985). This may have clinical significance in the chemotherapeutic effect of redox cycling agents. The iron binding properties of quinone

antitumour agents may be important in determining cellular iron availability. In a further part of this study, doxorubicin-ferrous iron (1:2) and mitozantrone-ferrous iron (1:2) were found to mediate oxidative damage of plasmid DNA in the presence of reduced glutathione. This is presumably due to the high affinity these compounds have to bind iron (reviewed by Garnier-Suillerot, 1988). However, AQ-iron mixtures (1:2) did not mediate DNA damage in this system. This indicated that these compounds do not form iron complexes. Since these compounds to not have aromatic hydroxyl groups, this indicates the importance of these groups in iron-binding by mitozantrone and doxorubicin. This was confirmed by the finding that a series of anthrapyrazole antitumour agents, including CI941, were found to require a 10-OH group in order to mediate strand scission of plasmid DNA in the presence of ferric iron and glutathione. Iron binding by doxorubicin and mitozantrone could be important in the mechanism of MCF-7 cell kill in several ways:-

(a) Plasmid DNA strand breakage by quinone-iron complexes is likely to be mediated via formation of reactive oxygen species, including hydroxyl radicals, by glutathione mediated redox cycling of drug-bound iron [see section 3.4.2]. Therefore, this process could possibly be involved in the formation of cellular DNA strand breakage. Furthermore, this redox cycling mechanism is independent of the ability of the quinone to undergo flavin reductase mediated redox cycling.

(b) Binding of adventitious cellular iron by compounds such as doxorubicin, with a high propensity to undergo flavin reductase mediated redox cycling, could provide iron for the Haber-Weiss reaction. In addition, the ketoaldehyde group of doxorubicin has been proposed to be able to reduce bound Fe(III) to Fe(II) hence propagating iron reduction independently of superoxide anions or glutathione (Gianni *et al.*, 1988).

These two possibilities are supported by the protective effect of the iron chelating agent ICRF-187 against doxorubicin-induced cardiotoxicity in cancer patients (reviewed by Myers *et al.*, 1986).

5.2 *The role of DNA binding in plasmid DNA strand breakage*

As described in section 5.1, doxorubicin, 1AQ, 1,5AQ and 1,8AQ produced strand breakage of plasmid DNA in the presence of purified cytochrome P/450 reductase. The mechanism of strand breakage is likely to involve enzyme mediated reductive activation of drug in a redox cycle process with resultant formation of hydroxyl radicals [see section 3.4.1]. The possible mechanisms of hydroxyl radical mediated strand scission have been described in section 3.4.1. However, it is difficult to ascertain whether drug-DNA binding is a prerequisite for strand breakage. Previous studies have shown that doxorubicin does not need to intercalate into DNA in order to mediate strand breakage in the presence of cytochrome P450 reductase (Berlin and Haseltine, 1981). Furthermore, studies have indicated that flavin reductases metabolically activate doxorubicin to a lesser extent when it is intercalated (Berlin and Haseltine, 1981; Kalyanaraman *et al.*, 1980; Peters *et al.*, 1986). In contrast, it has been shown that daunomycin can be reduced when intercalated (Rouscilles *et al.*, 1989). However, it is likely that at the high drug concentrations (50-100uM) used in the present study that drug surface binding to DNA will occur since intercalation sites will be saturated (section 1.16.1). Surface-bound drug may be a better substrate for reductases than free or intercalated drug. The enzymic reduction of bound drug could therefore lead to site specific formation of reactive oxygen species or drug alkylating species (Sinha and Gregory, 1981; Sinha, 1980; Wallace and Johnson, 1987) proximal to the DNA duplex [see section 3.4.1].

Similarly, the role of DNA binding in the mediation of plasmid DNA strand breakage by quinone-iron complexes in the presence of glutathione has not been fully elucidated. Doxorubicin-iron has been shown to form a ternary complex with DNA but it is not clear whether the iron in this complex can undergo glutathione mediated redox cycling [reviewed by Garnier-Suillerot, 1988]. It has been shown that doxorubicin-iron complex dissociates upon intercalation (Beraldo *et al.*, 1985). However, as discussed above, surface bound drug-iron complex may participate in glutathione mediated redox cycling resulting in formation of hydroxyl radicals and consequently site specific DNA strand breakage

[see section 1.9.2.1].

5.3 *The mechanism of cellular DNA strand breakage by quinone antitumour agents*

The preponderance of evidence in this study shows that mitozantrone, CI941 and 1,4AQ do not exert their MCF-7 DNA strand breakage and cytotoxicity via reductase mediated redox cycling. This implies the presence of an alternative mechanism of action. This study investigated the ability of quinone antitumour agents to inhibit topoisomerase activity, identified as topoisomerase I (see section 1.7.3), in an MCF-7 cell nuclear fraction. Doxorubicin (Tewey *et al.*, 1984) and mitozantrone (Tewey *et al.*, 1984; Crespi *et al.*, 1986) have been previously shown to be inhibitors of mammalian topoisomerase II (see section 1.7.2) with concomitant formation of topoisomerase II-DNA cleavable complexes which on treatment with protein denaturants result in double and single strand break formation. In the present study doxorubicin and all the AQ's were found to form MCF-7 topoisomerase I-DNA cleavable complexes which formed single strand breaks on treatment with sodium dodecyl sulphate (see section 3.4.3). Mitozantrone and CI941 were more potent than the above compounds and totally inhibited topoisomerase activity without forming such cleavable complexes. This was also seen at high doxorubicin concentrations [photograph 3.6). This effect can be attributed to a profound disruption of the torsion of the topoisomerase I plasmid DNA substrate resulting in an inability of the enzyme to bind to its substrate at the drug concentration used [section 3.4.3.1].

The significance of topoisomerase I cleavable complex *in vivo* is not clear. The strand breaks hidden by these complexes may not be critical in cell kill *per se*. It is likely that cellular processing of such complexes is required to convert them to a lethal event (see section 3.4.3.2). The inverse relationship between the cellular growth period after drug exposure and the LD50 concentration of mitozantrone and CI941 [see table 4.5] indicates the presence of a delay period prior to DNA strand breakage by mitozantrone and CI941 becoming lethal (see section

4.4.1). Cellular processing could include events such as DNA replication, DNA transcription and RNA and protein synthesis (reviewed by Bodley and Liu, 1988) [see section 1.7]. Furthermore, topoisomerases have been implicated in regulation of cellular gene and oncogene expression and topoisomerase I and II inhibitors have been shown to influence these processes [see section 1.7]. In addition, mitozantrone and CI941 have been shown to be potent inhibitors of DNA and RNA synthesis (Nishio and Uyeki, 1983; Showalter *et al.*, 1986). Topoisomerase I has been implicated in all these processes hence is intimately involved in cellular proliferation and the maintenance of the transformed phenotype of tumour cells (reviewed by Bishop, 1987). Consequently, topoisomerase cleavable complex formation by antitumour drugs is likely to result in a disruption of the cellular processes required for tumour cell proliferation. It has been shown that topoisomerase I and II cleavable complexes are formed reversibly and do not persist after removal of drug (Hsiang and Liu, 1989; Pommier *et al.*, 1984). However, agents such as mitozantrone and CI941 which are high affinity DNA binders may allow persistance of such complexes, long enough to allow cellular processing to a lethal event. This correlates with the more potent effect of mitozantrone and CI941 on MCF-7 topoisomerase I in comparison to the other agents in this study. Furthermore, this is supported by the increased cytotoxic potential of mitozantrone and CI941 despite producing a similar level of DNA strand breakage (at LD50 concentrations) as the other less cytotoxic quinones of this study. The increased cytotoxic potential of mitozantrone and CI941 may be related to the MCF-7 cellular DNA lesions (eg topoisomerase-DNA cleavable complexes) these agents produce being more difficult to repair than that caused by redox cycling agents (see section 1.9.2.1). Therefore, these lesions would be likely to persist in the genome. This would be aided by the strong DNA binding affinity of mitozantrone and CI941 which results in their persistance in the nucleus even after removal of drug from the extracelular milieu (see table 3.3). Furthermore, potent inhibition of DNA synthesis by mitozantrone and CI941 (Nishio and Uyeki, 1983; Showalter *et al.*, 1986) may result in inhibition of cellular repair processes when they persist in the nucleus. Collins and Johnson (1984) showed that DNA single strand breaks caused by clastogenic agents do not reseal in the presence of inhibitors of DNA synthesis. This is supported by the finding

doxorubicin and mitozantrone induced DNA strand breaks increase and persist after removal of drug [eg. Locher and Meyn, 1983; Deffie *et al.*, 1988; Broggini *et al.*, 1988].

1,4AQ does not redox cycle in MCF-7 cells therefore is likely to express MCF-7 DNA strand breakage and cytotoxicity via another mechanism. This could involve inhibition of topoisomerase I [see section 3.4.3]. However, this compound was not as cytotoxic as mitozantrone and CI941 in MCF-7 cells. This may be explained by 1,4AQ being a less potent inhibitor of MCF-7 topoisomerase I and having a lower binding affinity for DNA than mitozantrone and CI941 [table 3.3]. Formation of topoisomerase I-DNA cleavable complexes may contribute to the MCF-7 DNA strand breakage observed for doxorubicin, 1AQ, 1,5AQ and 1,8AQ. However, the strand breakage produced by doxorubicin, 1AQ, 1,5AQ and 1,8AQ is likely to be mainly due to reductase mediated redox cycling with resultant formation of hydroxyl radicals [see section 2.4.1 and 2.4.3]. As described above mitozantrone and CI941 were more cytotoxic in MCF-7 cells than these compounds indicating that DNA strand breakage caused by mitozantrone and CI941 is more lethal to the cell than oxidative damage. The decreased cytotoxicity of redox cycling quinones in relation to mitozantrone and CI941 may be explained in several ways:-

(1) The presence of intracellular protective mechanisms in the MCF-7 cell line such as glutathione, glutathione peroxidase, glutathione-S-transferase, superoxide dismutase and catalase that will protect MCF-7 cells against reactive oxygen formation by quinone antitumour agents (Sinha *et al.*, 1987; Deffie *et al.*, 1988). Only when these defences are overwhelmed will reactive oxygen formation become a significant problem since these mechanisms are designed to cope with the constant flux of oxygen radical formation during endogenous metabolic processes. However, reactive oxygen formation may be a problem in cells or tissues deficient in the above protective mechanisms (see section 1.10).

(2) Pryor, (1988) suggests that reactive oxygen formation brings about

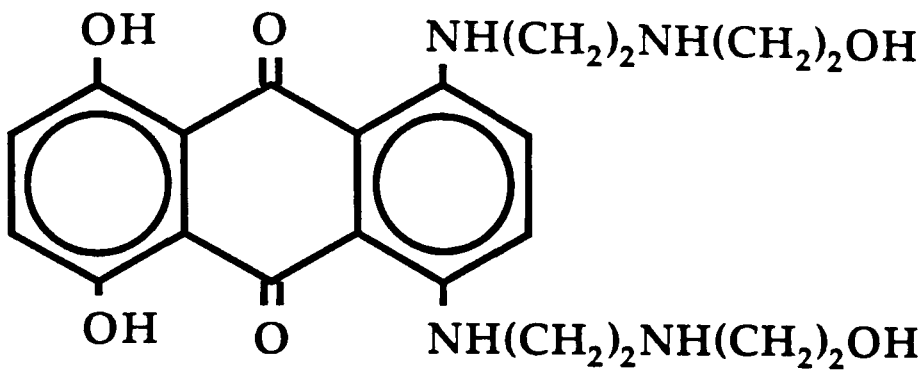
a limited number of types of DNA damage caused by the reactions of the hydroxyl radical. Consequently, this limited spectrum of damage can be enzymatically repaired in a number of processes that do not differ for each reactive oxygen generating system encountered. As described in section 1.9.2.3 many types of damage produced by the hydroxyl radical, including single strand breaks, are repairable by specific DNA repair enzymes. However, double strand breaks are more difficult to repair correctly since base information has been lost on both DNA strands [section 1.9.2.1]. Consequently, the formation of double strand breaks is thought to represent a more critical lesion with respect to cytotoxicity [Zijlstra *et al.*, 1987].

5.4 CONCLUSION

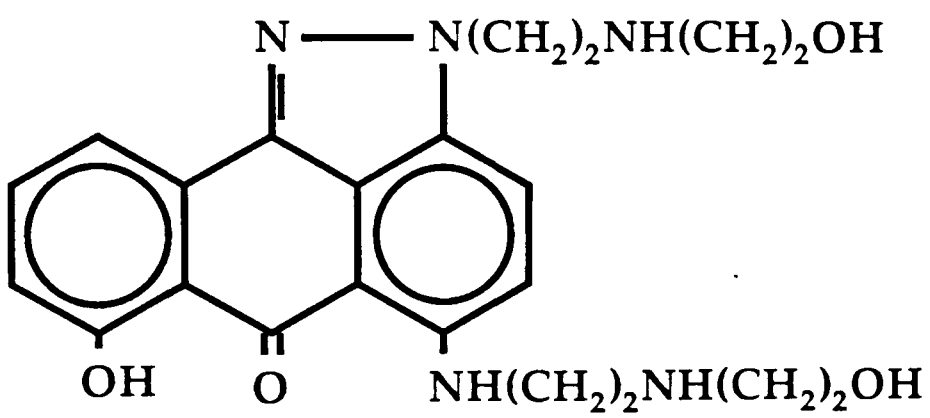
This study represents the first systematic investigation of the role of reductase mediated redox cycling of a series of related quinone antitumour agents in tumour cell kill. This study has found that reductase mediated redox cycling is likely to be involved in the DNA strand breakage and cytotoxicity of doxorubicin, 1AQ, 1,5AQ and 1,8AQ in MCF-7 human breast cancer cells. In contrast, despite the absence of redox cycling, mitozantrone and CI941 were considerably more cytotoxic in the MCF-7 cell line than the above agents. The cytotoxicity of mitozantrone and CI941 appears to be expressed, at least in part, via interaction with MCF-7 topoisomerase. 1,4AQ, an agent which does not redox cycle in MCF-7 cells, would appear to exert its cytotoxicity via a less potent effect on topoisomerase activity than mitozantrone and CI941.

In conclusion, despite the related anthraquinone based structure of the quinone antitumour agents in this study, these compounds possess different propensities with respect to their ability to undergo reductase mediated redox cycling, form cellular DNA strand breakage, bind iron, inhibit DNA topoisomerase I activity and kill MCF-7 cells. The mechanism of action of these agents appears to be intimately related

mitozantrone



CI941



1,4AQ

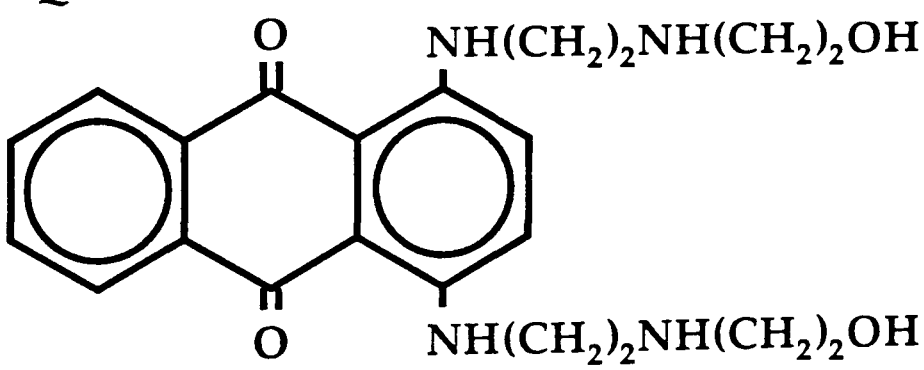


Figure 5.3 The structures of mitozantrone, CI941 and 1,4AQ

to their structural differences. The most important structural modification with respect to mechanism revealed in the present study appears to be the C_1 and C_4 positioning of the hydroxyethylamino side chains on the anthraquinone chromophore of mitozantrone, CI941 and 1,4AQ which is related to the absence of flavin reductase metabolic activation of these compounds. As described previously this may be related to steric hinderance between the drug molecule and the flavin component of the reductase (section 3.4.1). Another important structural modification is the aromatic hydroxyl groups on position 5 and 8 of the anthraquinone chromophore. Despite having identical side chains to mitozantrone and CI941, 1,4AQ does not have aromatic hydroxyl groups (see figure 5.3). The importance of these groups is reflected in the lower DNA binding affinity [see table 3.5], reduced inhibitory effect on MCF-7 topoisomerase I activity [section 3.4.3], reduced MCF-7 DNA strand breakage [section 3.4.4] and reduced MCF-7 cytotoxicity [section 4.4.1] of 1,4AQ in comparison to mitozantrone and CI941. This is supported by other studies which have found mitozantrone and 1,4AQ not to be metabolically activated (Basra, 1986; Kharasch and Novak, 1983a). However, mitozantrone is a more potent inhibitor of DNA synthesis (Nishio and Uyeki, 1983), more potent at forming cellular DNA strand breakage (Locher and Meyn, 1983) and more cytotoxic in cellular systems (Partridge, 1987). Furthermore, chromophore hydroxyl groups are important in determining the iron binding potential of mitozantrone and anthrapyrazoles [section 3.4.2]. The aromatic hydroxyl groups of mitozantrone and CI941 appear to be important in the enhanced MCF-7 cytotoxicity, DNA binding, topoisomerase I inhibition and cellular DNA strand breakage of these agents compared to the other quinone agents of this study. Clearly, subtle structural modification of the anthraquinone chromophore can be used to alter the activity of quinone antitumour agents. Since the non-redox cycling agents mitozantrone and CI941 were the most cytotoxic agents of this study it appears that the ability to produce free radical mediated effects confers no increased cytotoxic potential on agents such as doxorubicin and alkylaminoanthraquinones. This indicates that future development of quinone antitumour agents should concentrate on compounds which do not redox cycle. Furthermore, these agents should possess aromatic hydroxyl groups since these appear to be responsible for the enhanced cytotoxicity of mitozantrone and CI941 shown in the present study.

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Advanced studies undertaken in connection with the programme of research in partial fulfilment of the requirement of the degree of Doctor of Philosophy.

The candidate has presented communications at the Royal British Pharmaceutical Society Annual Conference in Manchester (September 1987), Aberdeen (September 1988) and Keele (September 1989). The candidate also presented a poster at the Royal British Pharmaceutical Society Annual Conference in Keele (September 1989). The candidate has also presented communications at The Society for Free Radical Research Satellite Meeting, Vienna (November 1988) and The British Association for Cancer Research and Association of Cancer Physicians Annual Conference, Glasgow (April, 1989). The candidate has also presented seminars within the Department of Pharmacy at Leicester Polytechnic and at the Department of Pharmacy , Sunderland Polytechnic.

Publications from this thesis

Fisher, G.R. and Patterson, L.H. (1987). Polarographic determination of mitozantrone uptake by MCF-7 human breast cancer cells. J. Pharm. Pharmacol. 39(suppl.). 48P. [Presented at Royal Pharmaceutical Society of Great Britain Annual Conference, 1987].

Fisher, G.R. and Patterson, L.H. (1988). Free radical generation by doxorubicin in MCF-7 human breast cancer cells. J. Pharm. Pharmacol. 40(Suppl.). 28P. [Presented at Royal Pharmaceutical Society of Great Britain Annual Conference, 1988].

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Appendix

Appendix

A1 Cell culture

A1.1 *Procedure for changing growth medium in a flask of cells*

Firstly the old medium was discarded and an appropriate quantity of fresh medium pipetted into the flask depending on its surface area [25cm^2-12ml, 100cm^2-20ml, 150cm^2-30ml]. Depending on the cell type the medium was gassed for 60 seconds with 95% air 5% CO_2. Generally the medium was changed every other day until confluence was reached (when cells covered >70% of flask surface).

A1.2 *Passage or sub-culture of cell lines*

The old medium was discarded, and EDTA [0.1% in DPBS, 10ml] was added and left in contact with the cells for 30 seconds. The EDTA solution was then discarded and the flask incubated (upright) at 37C for 10-15 minutes or until the cells slid off the flask surface. The cells were resuspended in fresh pre-warmed medium [5-10ml] and innoculated into flasks containing pre-warmed medium at a reduced cell concentration to the original flask, usually using a 1:2 or 1:3 fold dilution. If required the medium was gassed with 95% air/ 5% CO_2 for one minute. The flasks were incubated at 37C and checked regularly for confluency and contamination using an inverted microscope [Nikon TMS].

A1.3 *Preparation of large cell numbers using roller bottles*

Glass roller bottles [730cm^2, BDH] were used when large volumes of cells were required for preparation of subcellular fractions. The roller

bottles were initially sterilised by autoclaving, the neck of the bottle being covered by foil and the plastic caps being autoclaved separately.

Cell suspension which had been well dispersed (prepared from 1x150cm^2 confluent flask) was innoculated into the bottle with fresh medium (40ml). The bottle was rolled [using an RMV Universal Roller, Luckham] at a setting of 40% speed overnight and then the speed increased to 50-60% speed from then on. The medium needed to be replaced at least every other day.

A1.3.1 *Passage of cells in roller bottles*

EDTA solution [0.1%, 10mls] was placed in the bottle and the bottle rotated for one revolution at room temperature. The EDTA solution was then poured off and the bottle incubated for 10-15 minutes on the roller device at 37C. Following this the cells were resuspended in PBS/medium [20ml] by vigorously washing/rotating the sides of the bottle and the cell suspension removed. Residual cells in the bottle can be resuspended in growth medium [40ml] and the growth cycle re-initiated.

A1.4 *Preparation of RPMI-1640 medium*

L-Glutamine (200mM, 1ml), foetal bovine serum or Nuserum IV (10ml) and penicillin 5000 IUml^{-1}/ streptomycin 5000 ugml^{-1} (1ml) were added to a bottle of RPMI-1640 medium with Hepes buffer (20mM) without sodium bicarbonate or L-glutamine. All constituents were purchased from Flow Laboratories [UK].

A1.5 *Reconstitution of powdered RPMI-1640 medium*

Whilst gently stirring, RPMI 1640 powdered medium with Hepes buffer

(25mM) and L-glutamine, without sodium bicarbonate (1 litre sachet, Flow laboratories) was added to distilled water (900ml) and stirred until dissolved. The sachet was rinsed with a small volume of distilled water and this added to the above solution. The solution was made up to the final volume with distilled water and filter sterilised immediately through a filter disc [0.2um, millipore]. The sterile medium was aliquoted into sterile bottles (100ml) and incubated at 37C for 3 days to check for contamination.

A1.6 *Storage of cells in liquid nitrogen*

Storage of cell lines is an essential back up in the event of cell contamination and as a permanent store of cell lines not required in culture. The cells were prepared from culture as described above and resuspended in fresh medium (2ml) per confluent flask of cells. Dimethylsulphoxide [DMSO] was added to the suspension to a final concentration of 10%, the suspension mixed and aliquots (1ml) dispensed into 2ml plastic ampoules [Nunc]. The vials were placed in a polystyrene box, the box taped up and placed in a -20C freezer for 45 minutes. The box was then suspended in the vapour phase of an LR30A liquid nitrogen refrigerator (Union Carbide) for a further 45 minutes, the ampoules removed and placed in cylindrical ampoule straws (consisting of cardboard sleeve and metal holder). The straws were stored in a cryogenic refrigerator (10XT, Taylor Wharton).

A1.7 *Removal of cells from storage under liquid nitrogen*

The vial of cells was removed from the cryogenic refrigerator and placed in a 37C water bath. As soon as the cells were thawed [approx 5 minutes] the suspension was placed in a flask containing prewarmed growth medium [12ml]. The flask was incubated at 37C for 2 hours until the cells had adhered. The medium was then replaced to dilute the DMSO and the flask incubated at 37C.

A1.9 *The trypan blue assay for cellular viability*

Trypan blue solution (0.4%, 100ul) was added to cell suspension [250ul] in an eppendorf tube, mixed well and left for 1 minute. The surface of an improved Neubauer haemocytometer (depth 0.1mm) was moistened at the side of the counting chamber and the coverslip pressed into position, ensuring 'Newton's rings' were visible on both sides of the chamber. The counting chamber was filled with cell suspension using a Gilson pipette [10ul].

Non viable cells retain the trypan blue dye, appearing blue in colour, due to a disruption of membrane integrity. A differential count of viable to non viable cells was carried out using a microscope at low magnification [x20].

A1.9 *Counting of cells*

The haemocytometer counting chamber consisted of a marked grid pattern with a central area bound by triplicate lines which was $1mm^3$, this area being subdivided into 25 squares. The depth of fluid in the counting chamber was 0.1mm, therefore the total volume of liquid over the central area was:-

$$0.1x1x1 = 0.1\ mm^3 \text{ or } 10^{-4}ml$$

The total number of cells (n) over the central area or present in $o.1mm^3$ gives a concentration of:-

$$nx10^4$$

If the suspension was diluted, this was taken into account:-
eg. 250ul cells + 100ul trypan blue gives a x1.4 dilution, therefore the number of cells is given by:-

$nx10^4x1.4\ ml^{-1}$

A2.0 *Determination of protein content in subcellular fractions*

Protein determinations were carried out using the Biorad protein assay [Biorad] which utilises a solution of Coomassie blue dye. The Biorad solution was diluted 1:5 with distilled water and then filtered. A series of bovine serum albumin [BSA] concentrations were prepared [0.1-1.0mg ml^{-1}]. A series of triplicate test tubes were set up each containing stock BSA [100ul] and diluted Biorad solution [5ml]. The tubes were mixed well and allowed to stand for 10 minutes at room temperature, following which the absorbance of each tube was read immediately at 595nm against a blank [containing Biorad solution but no BSA]. A calibration curve was plotted and used to determine the protein concentration of samples [100ul] of 'unknown', treated as above. See figure A1 for a typical calibration curve.

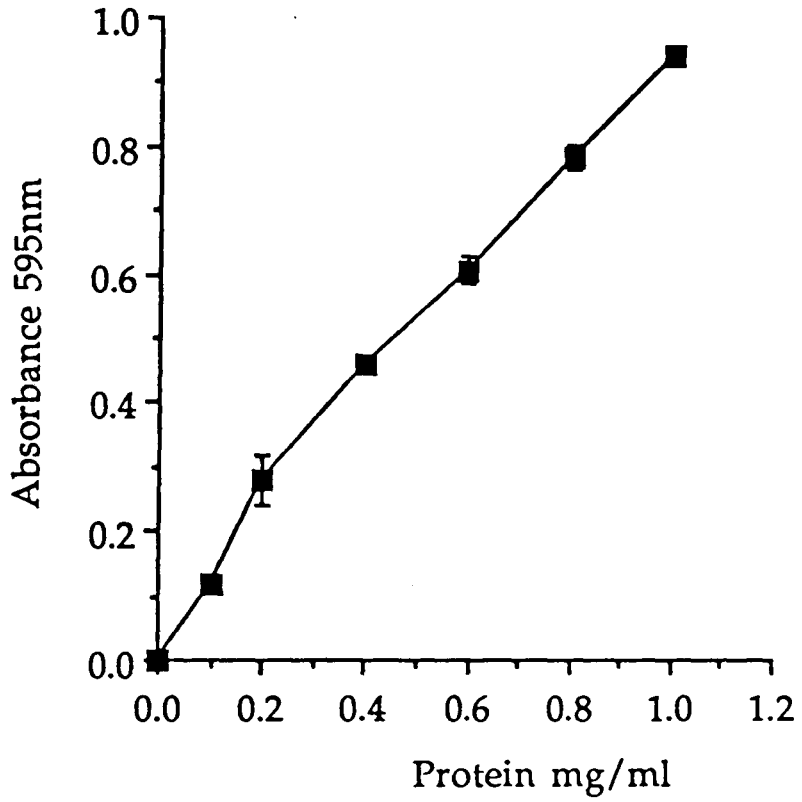


Figure A1 *A typical calibration curve for the Biorad protein assay.* Values are mean + sd of three determinations.

A3.0 *Preparation of acetylated cytochrome C*

Horse heart cytochrome C [100mg] was dissolved in sodium acetate solution [10ml, 50% saturated], keeping on ice. Under continuous stirring at 4C, ten times excess acetic anhydride with respect to the cytochrome c lysine residues was added [150ul] (one molecule of cytochrome c contains 19 lysine residues). The reaction was allowed to proceed for 30 minutes, following which, unreacted acetic anhydride was removed by dialysis using Spectrapor membrane tubing [molecular cut off 6-8K, diameter 14.6mm]. The membrane was soaked in running water for 4 hours prior to use, dialysis being carried out for 10 hours under cold running water.
The acetylated cytochrome c was stored under liquid nitrogen until required.

The extent of cytochrome c acetylation was determined using the ninhydrin reaction. A series of concentrations of native and acetylated cytochrome C were prepared [10-80uM]. Aliquots [1ml] of these solutions were placed in test tubes and ninhydrin reagent [0.5ml] added to each tube and vortexed. The tubes were covered in parafilm and placed in a boiling water bath for 15 minutes and then cooled on ice for a further 10 minutes. Ethanol [50%, 2.5ml] was added and the tubes vortexed. The absorbance of each tube was measured at 570nm against a blank. The extent of cytochrome c acetylation was calculated using the following formula:-

$$\% \text{ acetylation} = 100\left(1-\frac{\text{slope acetylated}}{\text{slope native}}\right)$$

The slopes being determined from a plot of absorbance at 570nm against concentration of cytochrome C [native and acetylated]. See figure A2 for an example of this plot. Cytochrome c concentration was determined spectrophotometrically using the extinction coefficient of 21mM cm^{-1} at 550nm [Williams and Kamin, 1962]. Before measuring the absorbance a few mg of sodium dithionite were added to the cuvette inorder to fully

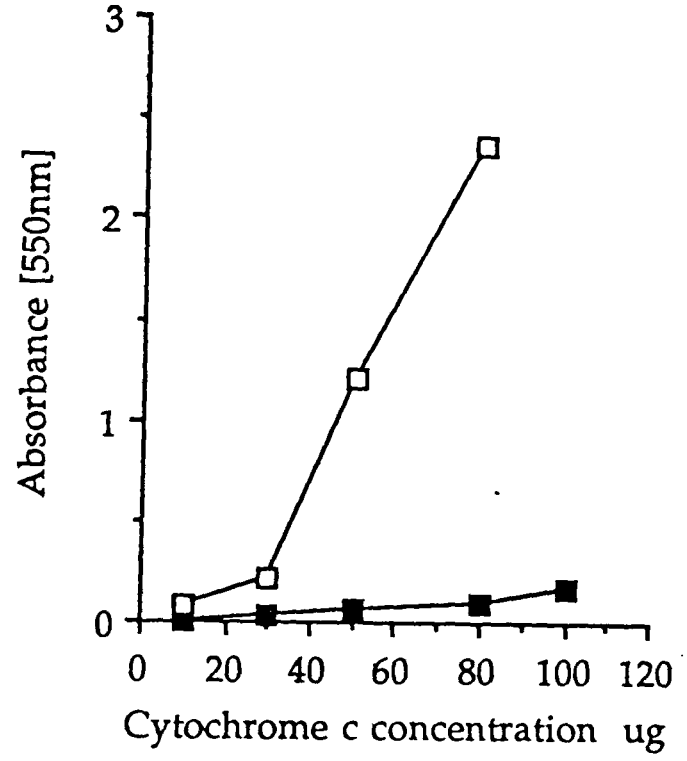


Figure A2 *Determination of the degree of acetylation of cytochrome c.*

$$\% \text{ acetylation} = 100\left(1-\frac{\text{slope acetylated [■]}}{\text{slope native [□]}}\right)$$

reduce the cytochrome c.

A4.0 *Preparation of agarose gel for submarine gel electrophoresis*

Agarose [1g] was added to TAE buffer [110ml] and the solution microwaved at medium setting for 5 minutes. The molten agarose solution was poured into a gel mould, consisting of a horizontal perspex plate [12.5 x 11.5cm] with temporary side walls constructed from adhesive tape. A perspex comb was inserted into the molten gel in order to form the sample wells. Once the gel had set the sample comb and temporary walls were removed and the gel plate was fully immersed in an electrophoresis tank filled with TAE buffer (800ml).

A5.0 *Reconstitution of powdered anthraquinone antitumour agents*

A vial of the agent [HCl salt] was opened and a spatula full of the powder was transferred to a larger sealable vial [5ml]. A volume of distilled water was added and the vial sealed and crimped immediately. The concentration of drug was determined by measuring the absorbance of drug [10ul in 1ml distilled water] at the appropriate wavelength and calculation using the corresponding extinction coefficient [see below]. The drugs were stored at 4C at a concentration of 5-10mM in light protected vials.

Table A1 *Extinction coefficients and absorption maxima for anthraquinone antitumour agents*

Drug	Absorption max [nm]	Extinction coefficient [mM cm^{-1}]
doxorubicin	495	13.0
mitozantrone	658	20.9
1AQ	498	6.23
1,4AQ	583	15.0
1,5AQ	518	11.5
1,8AQ	540	8.45
CI941	492	19.315

A6.0 *Preparation of NADPH*

Several mg of NADPH was dissolved in distilled water [200-500ul] The concentration of NADPH was determined by measuring the absorbance at 340nm [10ul in 1ml distilled water] and calculation using the extinction coefficient of 6.3mM cm^{-1} [Handa and Sato, 1976]. The prepared stock solution was used on the same day and then discarded.

A7.0 *Preparation of cultured cell S9 homogenate*

The cultured cells were isolated (see section A1.0), suspended in a small volume of ice cold DPBS (10^7 ml^{-1}), sonicated for 5 minutes, and centrifuged at 10,000 rpm for 30 minutes. The supernatant (S9 fraction)

was collected and was stored in 0.5-1ml aliquots under liquid nitrogen if not required immediately.

A8.0 *Preparation of denatured calf thymus DNA*

Calf thymus DNA [30mg, sodium salt, Sigma] was added to single strand eluting solution [90ml, pH12.3] in a volumetric flask [100ml]. The solution was left at room temperature overnight in order for the DNA to dissolve. The volume was made up to 100ml with eluting solution. The DNA was denatured by placing in a capped plastic universal tube [50ml] and heating in a water bath [60C] for one hour.

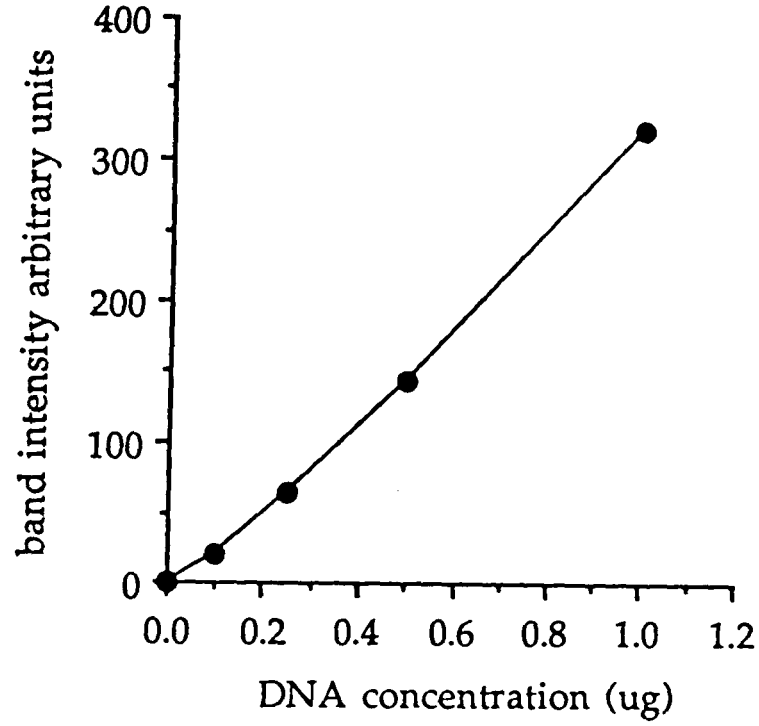


Figure A3 *Calibration curve for the detection of supercoiled plasmid DNA on agarose gels.*

The gel was photographed and the intensity of the DNA bands on the negative determined by laser densitometry [as described in section 3.2.1]. Values are mean + sd of three determinations.

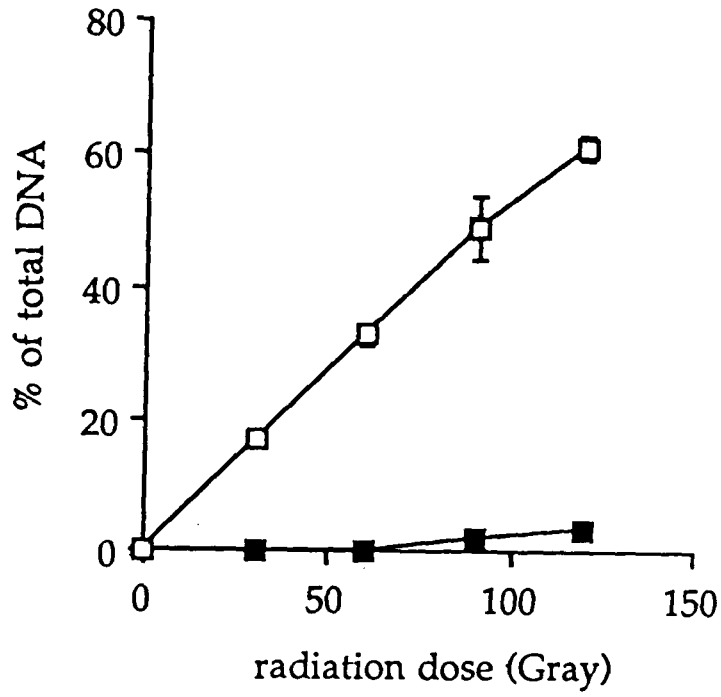


Figure A4 *Effect of gamma radiation on strand breakage of plasmid DNA.*

Supercoiled plasmid DNA (300ng) was exposed to gamma radiation at room temperature as described in section 3.2.4.2. The DNA species resolved by gel electrophoresis were open circular [□] and linear [■]. Values are mean + sd of three determinations.

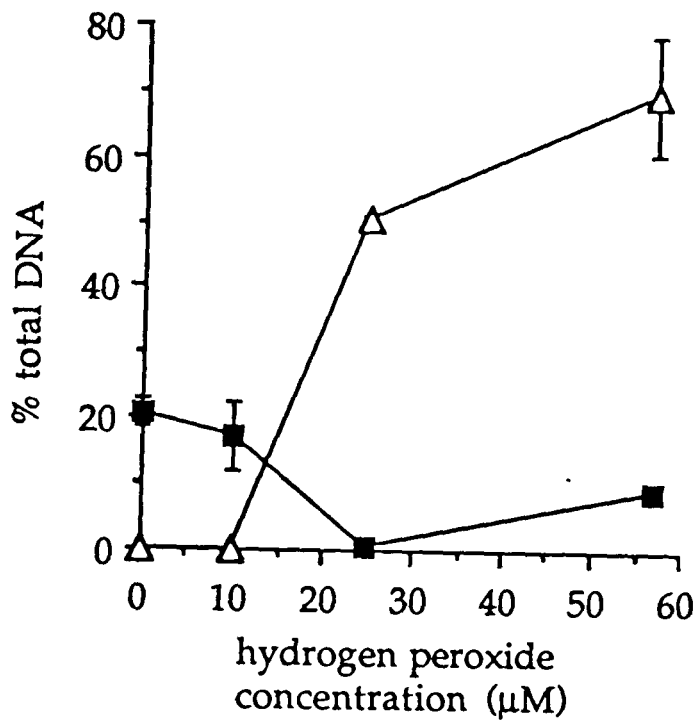


Figure A5 *Effect of hydrogen peroxide on strand breakage of plasmid DNA.*

DNA species resolved by gel electrophoresis were open circular [■] and linear [△].

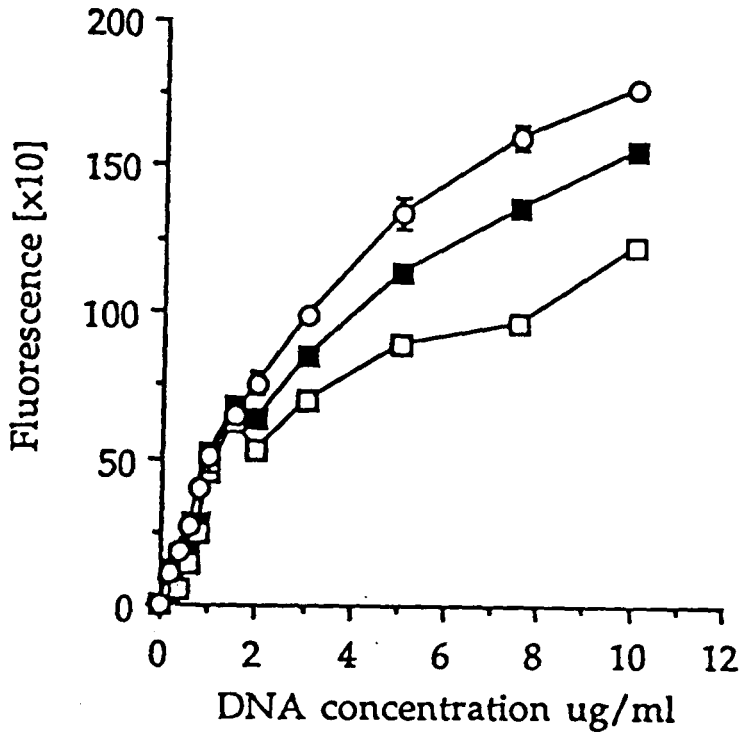


Figure A6 *Calibration curve for the detection of denatured calf thymus DNA using Hoechst H33258.*

Hoechst concentration was 50nM [□], 150nM [■] and 450nM [○]. Values are mean + sd of three determinations.

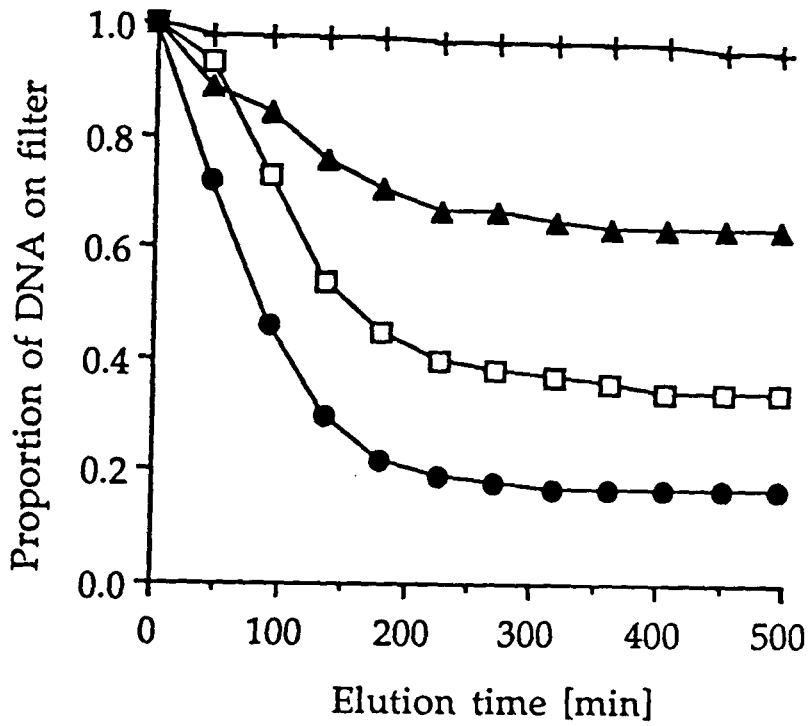


Figure A7 *Elution profile of DNA isolated from MCF-7 cells treated with gamma radiation.*

MCF-7 cells [$10^6 ml^{-1}$] were untreated [+] or exposed to 2 Gray [▲], 4 Gray [□] or 8 Gray [●] gamma radiation at 0C [as described in section 3.2.4.]

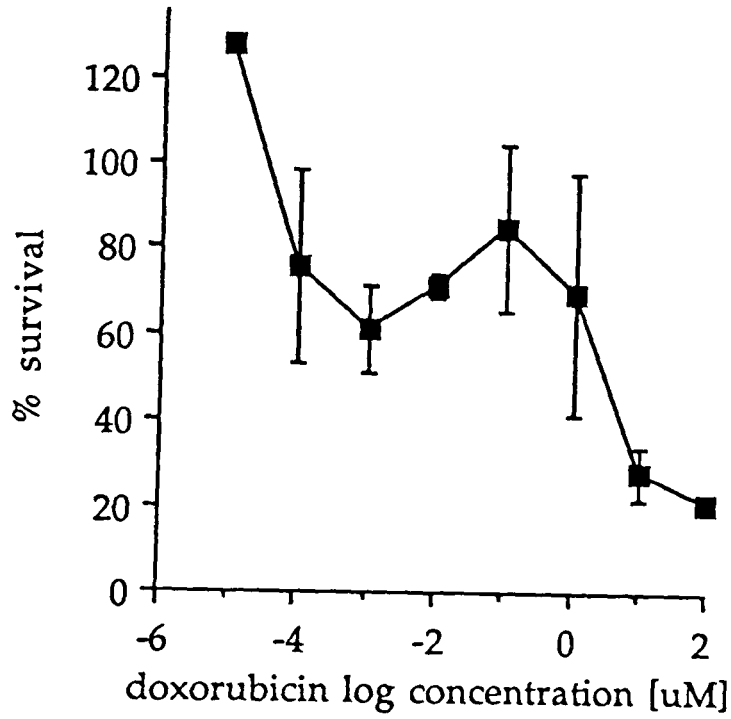


Figure A8 *Cytotoxicity of doxorubicin in MCF-7 cells following a one hour exposure and 6 day further growth period.*

For experimental details see figure 4.8. Values are mean + sd of three determinations.

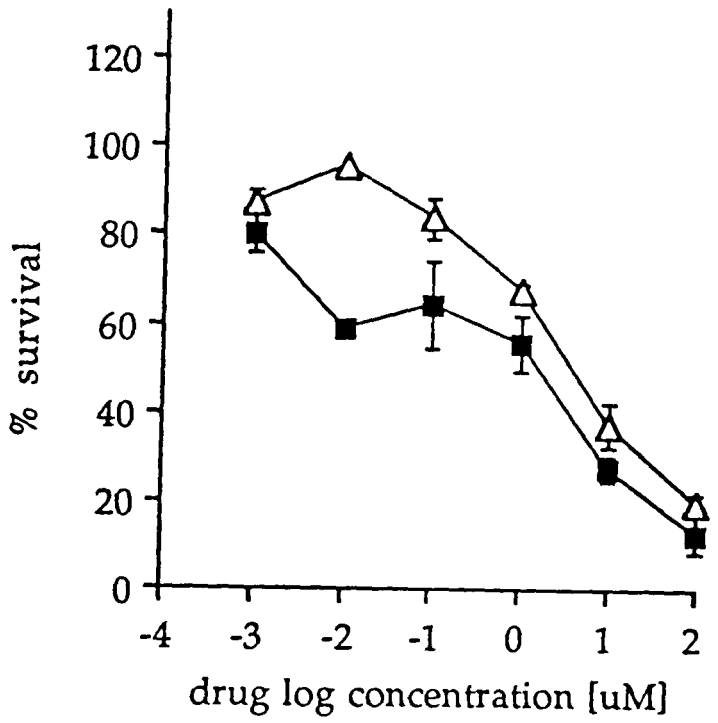


Figure A9 *Cytotoxicity of mitozantrone [■] and CI941 [△] in MCF-7 cells following a 24 hour exposure.*

For experimental details see figure 4.7A. Values are mean + sd of three determinations.

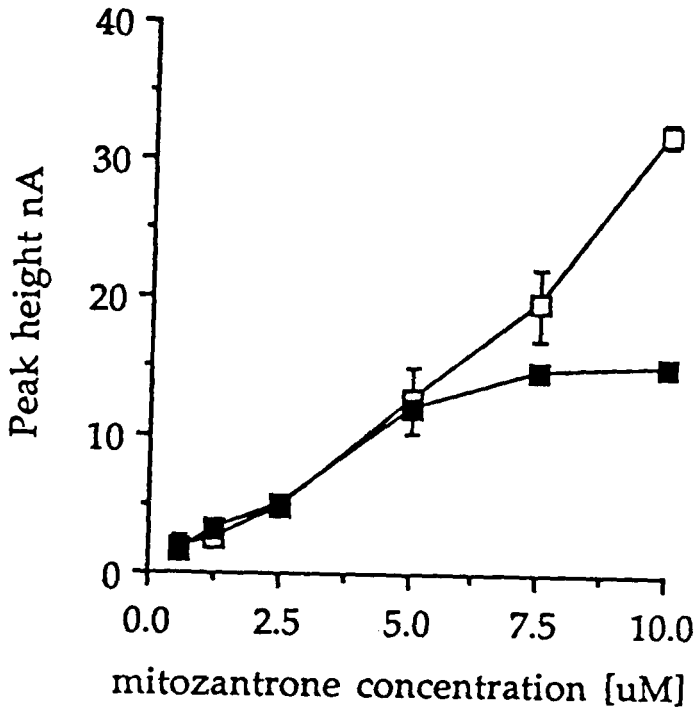


Figure A10 *Calibration curve for the polarographic determination of mitozantrone.*

Peaks are -1.04V [■] and -0.94V [□]. Values are mean + sd of three determinations.

Addendum

An investigation of the oxidative activation of quinone antitumour agents by horseradish peroxidase

Addendum- An investigation of the oxidative activation of quinone antitumour agents by horseradish peroxidase

Contents

List of figures, tables and photographs.

1 Introduction

Peroxidase mediated oxidative activation to reactive intermediates has been implicated in the toxicity of a wide range of xenobiotics (reviewed by Mason and Chignell, 1982). Figure 1 shows the proposed mechanism of action of horseradish peroxidase (HRP) on a substrate X. Chapter 2 of the present study has investigated the propensity of doxorubicin, mitozantrone, CI941 and the alkylaminoanthraquinones to undergo reductive activation by reductases in the process of redox cycling. Furthermore, chapter 3 has investigated the ability of these agents to mediate damage to cellular DNA in the presence of purified reductases. However, oxidative activation of these agents to free radical species which could damage DNA has not been previously considered. In the present study the propensity of mitozantrone to undergo oxidation by HRP was investigated. In addition, studies on the ability of these agents to produce DNA strand breakage in the presence of HRP were carried out.

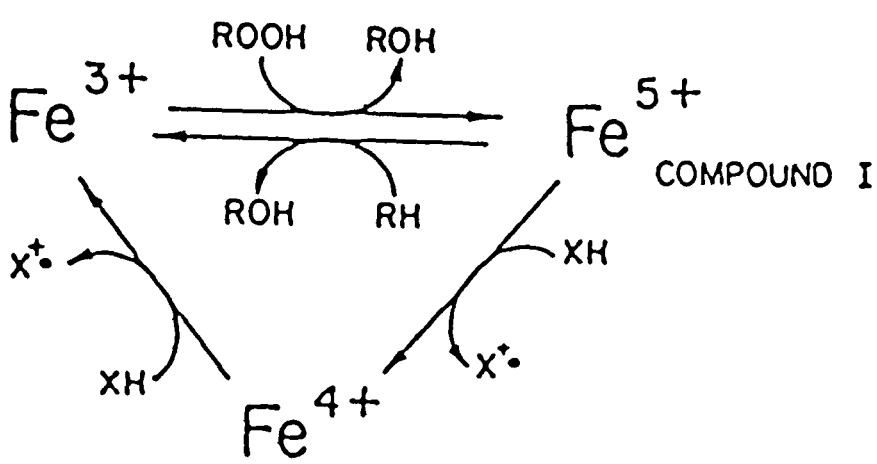


Figure 1 *Peroxidase oxidation states and peroxidase mechanism.*

X= co-substrate
ROOH= hydroperoxide cofactor

(Ross, 1988)

2 Methods

2.1 *Spectral studies on the effect of mitozantrone on HRP compound II formation*

Spectral studies on the interaction of mitozantrone with HRP Compound I were carried out following the addition of hydrogen peroxide (2uM) to HRP (2.4uM) at 37C. Spectral changes were followed using a uv/vis scanning spectrophotometer [552 spectrophotometer, Perkin Elmer] fitted with a temperature controller and temperature controlled Peltier cell [Perkin Elmer].

2.2 *Determination of strand breakage of supercoiled plasmid DNA by quinone antitumour agents in the presence of HRP and hydrogen peroxide*

The technique was carried out in an identical manner as described in section except the incubate consisted of $MgCl_2$ [3mM], Tris buffer[100mM, pH 7.0], DNA [300ng], hydrogen peroxide [0.05uM], horse radish peroxidase [0.036ug] and drug [0-50uM]. The above components were incubated for 45 minutes at 37C.

2.3 *Esr studies on free radical generation by quinone antitumour agents in the presence of HRP and hydrogen peroxidase.*

Esr studies were carried out as described in section except the incubate consisted of drug (3.28mM), horse radish peroxidase (56ug ml^{-1}), hydrogen peroxide (2.26mM) and phosphate buffer (100mM, pH 7.4).

3 Results

3.1 *The effect of mitozantrone on conversion of HRP compound I to compound II*

Figure 2 shows that shows that the addition of hydrogen peroxide to HRP resulted in a bathochromic and hypochromic shift from the absorbance maximum of HRP [Fe(III), 403nm] to that characteristic of HRP compound I [410nm]. Compound I was unstable and slowly converted to HRP compound II [418nm]. When a stoichiometric amount of mitozantrone was added this conversion was accelerated. When excess mitozantrone was added HRP [Fe(III)] was regenerated. These results indicated that one and/or two electron oxidation of mitozantrone was being carried out by HRP.

3.2 *The effect of mitozantrone on plasmid DNA in the presence of HRP and hydrogen peroxide.*

Figure 3 [data derived from photograph 1] shows that mitozantrone (10uM) produced an increase in single strand breakage relative to the control in this system. It can be seen that mitozantrone (25uM) produced a total loss of the DNA present, whist mitozantrone (50uM) produced no significant strand breakage relative to the control. The loss of DNA observed with 25uM mitozantrone was further investigated (figure 4). This figure shows that mitozantrone at this concentration caused total loss of the plasmid DNA in this system. However, all other combinations of the components of this system produced no effect indicating that the presence of the complete system containing mitozantrone was required for the total loss of DNA observed. At mitozantrone [25uM] a blue precipitate was observed in the incubation tube following ethanol precipitation and drying.

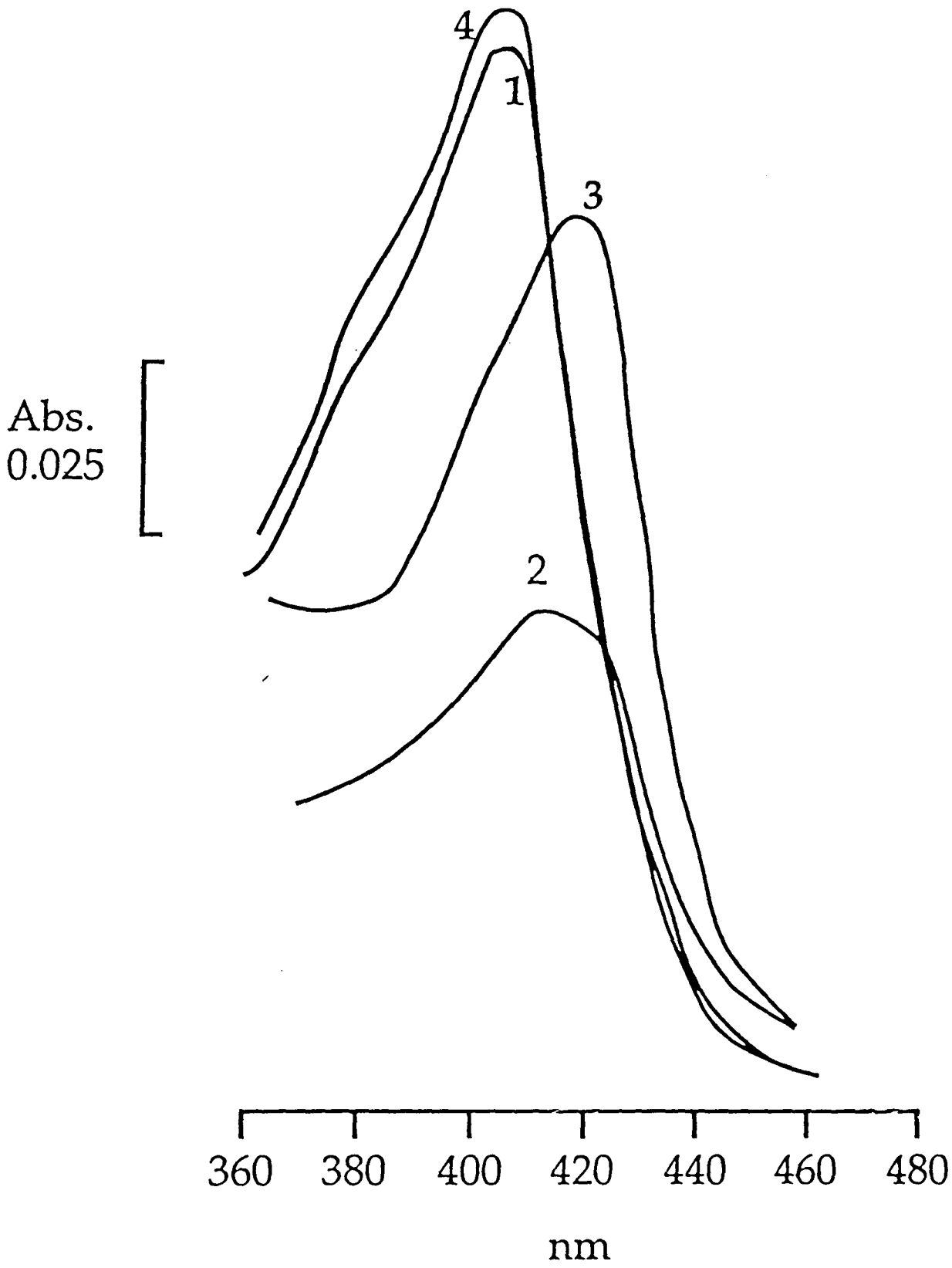
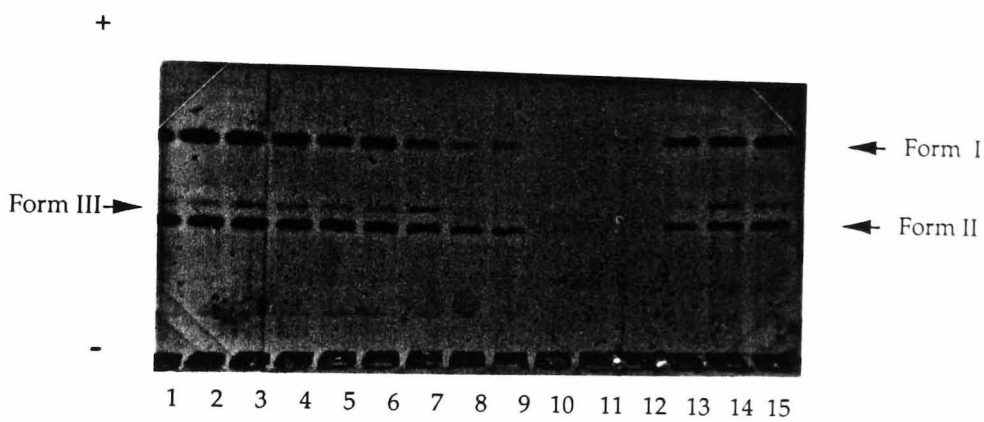


Figure 2 *Spectral changes of HRP in the presence of mitozantrone.*

1. Spectrum of HRP[Fe(III)] (0.72uM); 2. Spectrum of HRP after addition of hydrogen peroxide (1.2uM); 3. Spectrum of HRP compound II after addition of mitozantrone (0.25uM); 4. Spectrum showing regeneration of HRP[Fe(III)] after addition of excess mitozantrone (2.25uM).



Photograph 1 *Effect of mitozantrone on plasmid DNA in the presence of horseradish peroxidase and hydrogen peroxide.*

Plasmid DNA (300ng) was incubated (37C, 45min) with HRP (0.036ug) and hydrogen peroxide (0.05uM [lanes **1-3**], and mitozantrone 5μM [**lanes 4-6**], 25μM [**lanes 7-9**], and 50μM [lanes **13-15**].

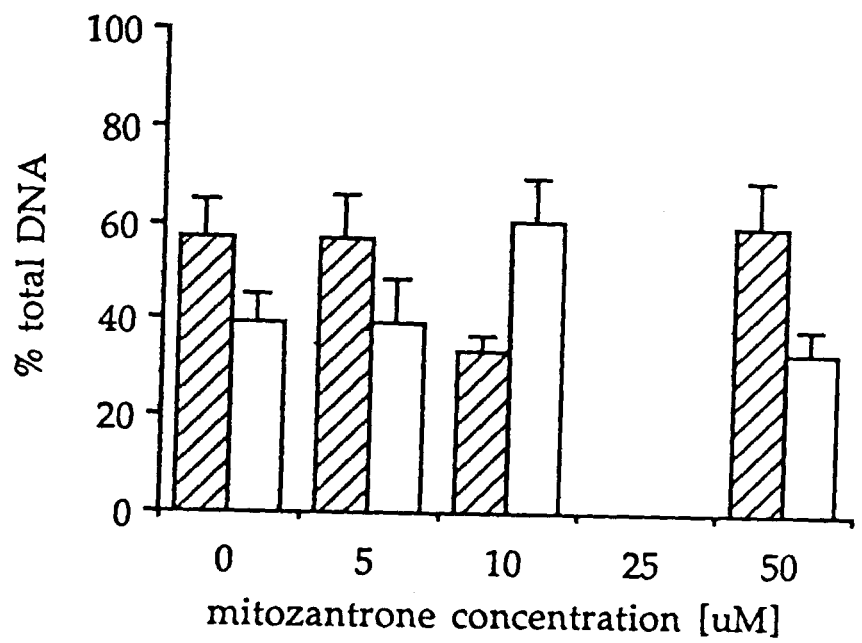


Figure 3 *Effect of mitozantrone on plasmid DNA in the presence of HRP and hydrogen peroxide.*

DNA (300ng) was incubated (37C, 45min) with HRP (0.036ug), hydrogen peroxide (0.05uM) and mitozantrone. The DNA species resolved by gel electrophoresis were supercoiled [▨] and open circular [□]. Values are mean + sd of three determinations.

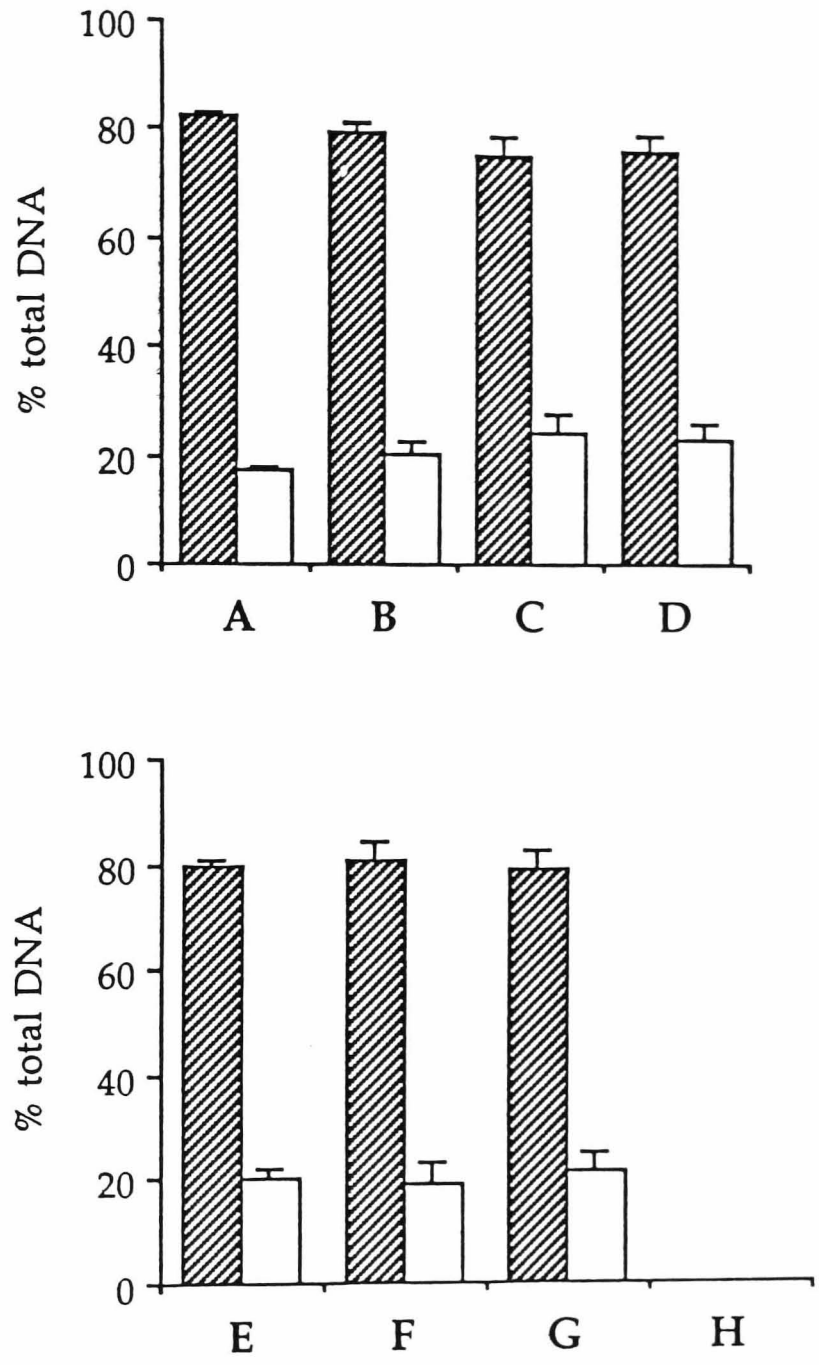


Figure 4 *Effect of mitozantrone on plasmid DNA in the presence of HRP and hydrogen peroxide.*

DNA (300ng) [**A**] was incubated (37C, 45min) with HRP (0.036ug), hydrogen peroxide (0.05uM) and mitozantrone (25uM) in the following combinations:- HRP [**B**], H_2O_2 [**C**], HRP and H_2O_2 [**D**], HRP and mitozantrone [**E**], mitozantrone and H_2O_2 [**F**], mitozantrone [**G**] and HRP, H_2O_2 and mitozantrone [**H**]. DNA species resolved by gel electrophoresis were supercoiled [▨] and open circular [□]. Values are mean + sd of three determinations.

3.3 *The effect of doxorubicin, CI941 and alkylaminoanthraquinones on plasmid DNA in the presence of HRP and hydrogen peroxide.*

Figure 5 shows that doxorubicin and CI941 (25 and 50uM) produced no significant DNA strand breakage or loss of DNA in a system of HRP/H_2O_2 relative to controls. The effect of AQ,s in this system was also investigated (figure 6). It can be seen that none of these compounds (25uM) produced significant strand breakage of DNA relative to controls.

3.4 *Esr studies on free radical formation by mitozantrone, doxorubicin and CI941 in the presence of HRP and hydrogen peroxide.*

These studies were carried out as described in section 2.3. Figure 7 shows that a free radical with a peak to peak width of 20 Gauss was detected in the presence of mitozantrone in this system. This signal was dependent on the presence of HRP, H_2O_2 and mitozantrone. However, in the presence of doxorubicin or CI941 under identical experimental conditions no esr signal could be detected.

4 Discussion

4.1 *Oxidative activation of mitozantrone by HRP.*

Mitozantrone produced characteristic spectral changes in the presence of HRP/H_2O_2 indicative of mitozantrone being a substrate for one or two electron oxidation by this enzyme (figure 2). By analogy with the known action of HRP on other substrates (figure 1) formation of a mitozantrone free radical species is likely to occur. This was confirmed by esr studies on a system of mitozantrone, HRP and hydrogen peroxide which showed the formation of a broad esr spectrum. This signal is likely to represent a polymeric mitozantrone free radical species which rotates slowly on the esr time scale leading to the esr

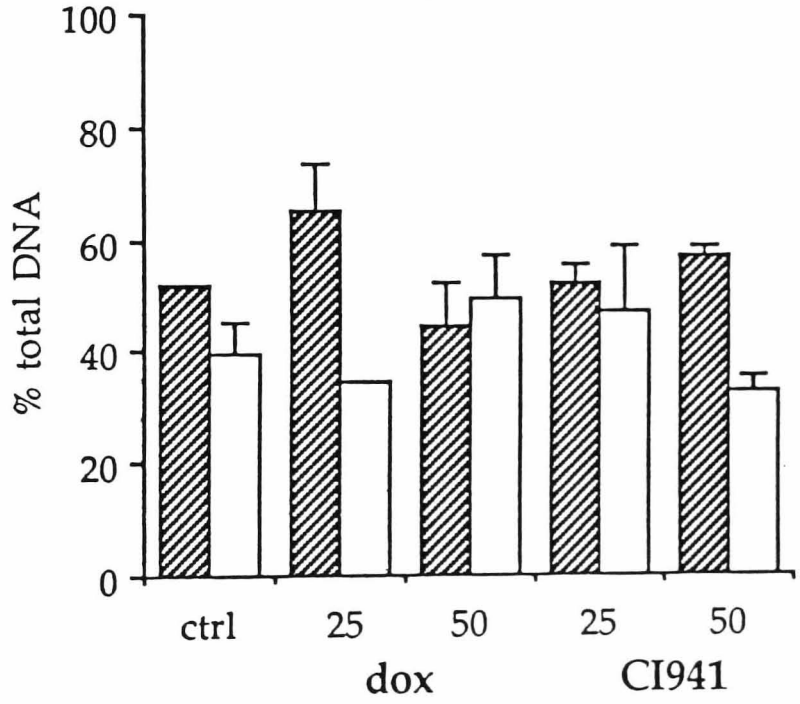


Figure 5 *Effect of doxorubicin and CI941 on plasmid DNA in the presence of HRP and hydrogen peroxide.*

Experimental conditions were as described in figure 3 (concentration in uM). DNA species resolved by gel electrophoresis were supercoiled [▨] and open circular [□]. Values are mean + sd of three determinations.

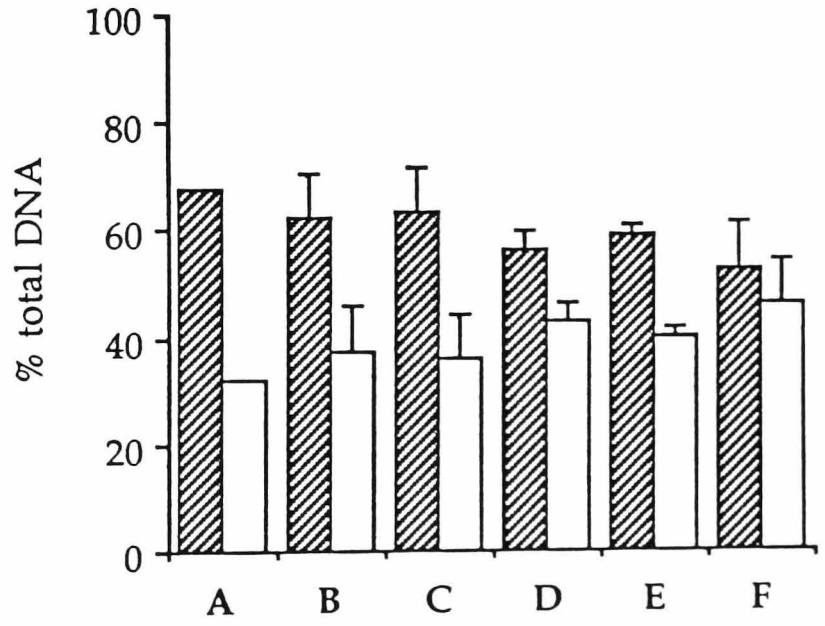


Figure 6 *Effect of alkylaminoanthraquinones (25uM) on plasmid DNA in the presence of HRP and hydrogen peroxide.*

DNA (300ng) [A] was incubated (37C, 45min) with HRP (0.036ug), H_2O_2 (0.05uM) [B] and 1AQ [C], 1,4AQ [D], 1,5AQ [E] and 1,8AQ [F]. DNA species resolved by gel electrophoresis were supercoiled [▨] and open circular [□]. Values are mean + sd of three determinations.

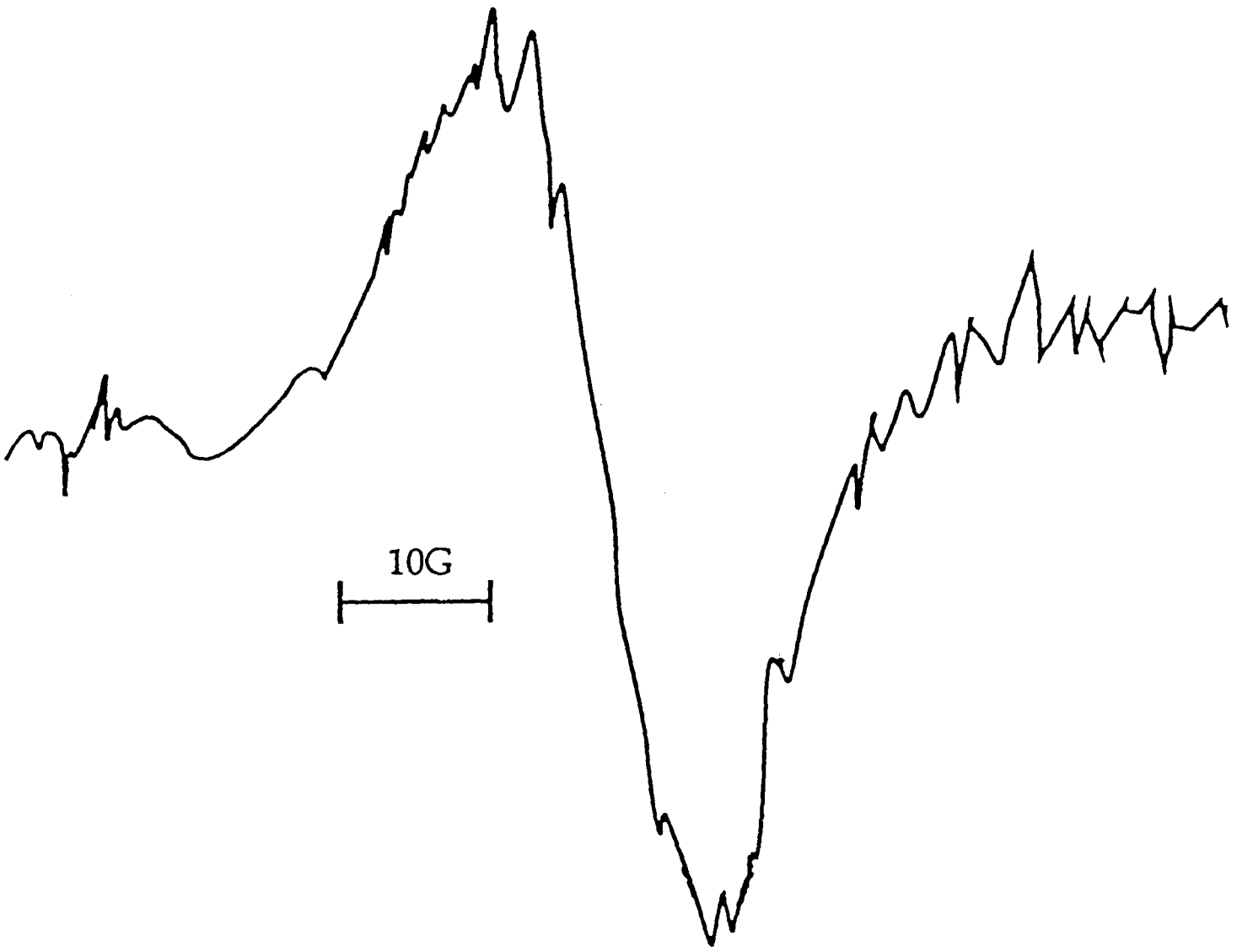


Figure 7 *Free radical formation by mitozantrone, in the presence of HRP and hydrogen peroxide.*

Incubate consisted of HRP (56ug ml^{-1}), hydrogen peroxide (2.26mM) and mitozantrone (3.28mM). Esr parameters were as described in figure 2.7A.

line broadening observed (figure 7). In the presence of HRP/H_2O_2, mitozantrone produced concentration dependent single and double plasmid DNA strand breaks [1 and 5uM] (figure 3). Furthermore, at 25uM, mitozantrone produced a total destruction of the DNA species present [figure 3 and 4 photograph 1]. Also at this concentration a blue precipitate was observed in the incubation tubes after the ethanol precipitation and drying stage. This indicates that mitozantrone is forming DNA-mitozantrone crosslinks which are likely to be too bulky to be resolved by gel electrophoresis. Mitozantrone (50UM) appeared to have no effect on plasmid DNA [figure 3]. This is possibly due to formation of mitozantrone dimers at high drug concentration which are formed in competition to DNA interaction. The DNA damage caused by this HRP/H_2O_2/mitozantrone system provides further evidence for formation of a mitozantrone free radical intermediate. A schematic diagram for the activation of mitozantrone by HRP is shown in figure 8. The oxidation of mitozantrone by HRP in the present study is supported by the work of Kolodziejczyk *et al.* (1988) and Reszka *et al.* (1986) who showed that mitozantrone is oxidised by HRP to a cyclised cationic free radical species due to one electron oxidation of the aromatic nitrogen atoms. However, these workers did not investigate whether this species could mediate DNA damage.

The results presented here have important implications for the mechanism of action of mitozantrone. This is because peroxidase enzymes are widely distributed in mammalian systems [see table 1] and offer a means by which mitozantrone could exert its antitumour or toxic effects. Further studies are required to investigate whether mitozantrone can undergo oxidative activation by more physiological relevant peroxidases.

4.2 *Oxidative activation of doxorubicin, CI941 and alkylaminoanthraquinones by HRP.*

In contrast to mitozantrone, doxorubicin (figure 5), CI941 (figure 5) and AQ's (figure 6) did not produce strand breakage in the presence of HRP/H_2O_2. Furthermore, doxorubicin and CI941 did not form free radical species in this system as studied by esr. This indicates that these

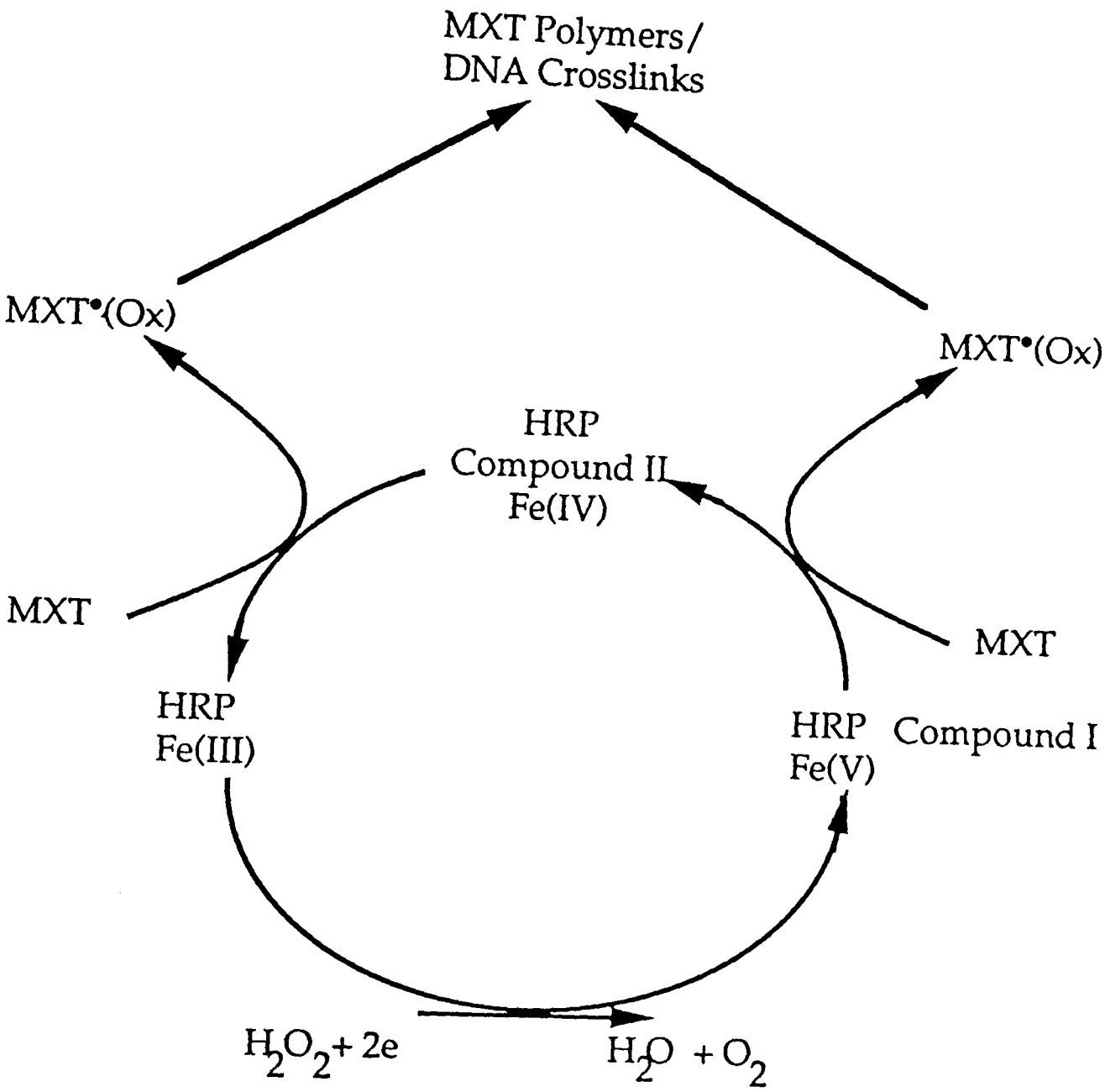


Figure 8 *A schematic diagram for the oxidation of mitozantrone by horseradish peroxidase*

compounds are not substrates for HRP, suggesting that oxidative activation does not play a role in their mechanism of action. In support of this, Kolodziejczk *et al.* (1989) showed that daunorubicin did not undergo oxidation by HRP.

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Peroxidase	Location	Pharmacological/ toxicological significance
Myeloperoxidase	bone marrow	benzene-induced myelotoxicity
	phagocytic cells	antimicrobial
Uterine peroxidase	uterus	diethylstilbestrol-induced genital tract tumours
Prostaglandin-H-synthetase	wide distribution localised in seminal vesicles, kidney medulla and bladder	prostaglandin synthesis aromatic amine induced bladder cancers
Lactoperoxidase	milk, saliva, lacrimal fluid	antimicrobial
Thyroid peroxidase	thyroid	iodide oxidation thyroxine formation
Cytochrome P-450	wide distribution	

Table 1 *Peroxidase systems*